EXTENSIVE INVESTIGATION OF RETICULOENDOTHELIOSIS VIRUS IN THE ENDANGERED ATTWATER'S PRAIRIE CHICKEN

A Dissertation

by

RYAN LANIER BOHLS

Submitted to the Office of Graduate Studies of
Texas A&M University
in partial fulfillment of the requirements for the degree of

DOCTOR OF PHILOSOPHY

May 2006

Major Subject: Veterinary Microbiology

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Approved by:

Chair of Committee,	Ellen Collisson
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ABSTRACT

Extensive Investigation of Reticuloendotheliosis Virus in the Endangered Attwater's Prairie Chicken. (May 2006)

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Chair of Advisory Committee: Dr. Ellen Collisson

Reticuloendotheliosis virus (REV) is a retrovirus that causes a neoplastic disease in a wide range of avian hosts including chickens, turkeys, and ducks. In 1993, REV was detected in the endangered Attwater's prairie chicken (*Tympanachus cupido attwateri*), a subspecies of *Tympanachus cupido*. Subsequent infections of this prairie chicken have been identified at captive breeding facilities throughout Texas. The implications of these infections have severely hindered repopulation efforts by these facilities. This study focused on investigating REV infection of captive Attwater's prairie chicken in order to better understand the disease affecting these endangered birds. The overall objective was to develop a means of eliminating this threat to the repopulation of the Attwater's prairie chicken. Several aspects of virus infection were investigated. Reagents capable of recognizing prairie chicken IgY and viral gag polypeptides were developed for use in assays for detection of antibody responses and titration of viral concentrations. Sequencing data of genomes collected from isolates of Texas prairie chickens and domestic chickens, as well as three REV prototype viruses, were compared to determine relationships among strains and identify the potential origin of the REV infecting Attwater's prairie chicken. Additionally, a flow cytometry

technique of segregating the lymphocyte population from peripheral blood mononuclear cells (PBMC) using a pan leukocyte monoclonal antibody was developed to more accurately measure changes within lymphocyte populations. This technique combined with intracellular labeling was used to deduce the target cells of REV infection. A nested polymerase chain reaction (PCR) test was developed for greater sensitivity in detecting infection in birds than the previous method of single amplification PCR. This greater sensitivity results in earlier identification of the virus in infected birds, which allows for earlier removal of infected birds to minimize transmission of the virus throughout the flock. The sensitivity of the nested PCR diagnostic test was determined in a dose response pathogenesis study, which was conducted on hybrid greater/Attwater's prairie chicken to observe the experimental development of disease in these birds. Finally, a vaccine was developed using plasmid DNA with REV encoded genes and tested on naturally infected prairie chickens to determine its efficacy in reducing viral load. Although no reduction in viral load was detected, the vaccine may be effective in providing prophylactic protection in future studies.

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INTRODUCTION

PRAIRIE CHICKENS

Tympanuchus cupido, one of two species of prairie chickens, was once widely distributed throughout the United States. Three subspecies of *Tympanuchus cupido* existed, including the greater prairie chicken (GPC; *T. c. pinnatus*), the Attwater's prairie chicken (APC; *T. c. attwateri*), and the heath hen (*T. c. cupido*) (Aldrich, 1963). These North American birds once roamed most of the United States and lower Canada. The heath hen occupied the northeastern part of the United States as far south as Virginia. Loss of coastal prairie habitat due to human development caused a decline in the population (Aldrich, 1963), which was subsequently hunted to near extinction. In 1907, the remaining 50 birds were put in a 648 hectare sanctuary on Martha's Vineyard, MA. The numbers grew to 2,000 until a fire swept through the sanctuary depriving the birds of food and cover, exposing the birds to hunger and predators. The decimated population then fell victim to disease introduced to the island by domestic turkeys (Gross, 1928). The heath hen went extinct in 1932 (Gross, 1963). The fate of the heath hen may represent a common sequence of events that leads to extinction.

This dissertation follows the format of *Virology*.

Currently, the Attwater's prairie chicken faces many of the same problems once encountered by the heath hen. The natural habitat of the Attwater's, once numbering in the millions, encompassed the gulf coastal plain of Texas and Louisiana. An initial decline in the Attwater's population, as a result of over hunting, has been followed by urban development and agricultural expansion of this region within the last century leading to a severe loss of habitat and a resulting decrease in the numbers of Attwater's prairie chickens. In 1967, the Attwater's prairie chicken was placed on the Federal Endangered Species List (Ehrlich, 1992; Johnsgard, 1983).

Captive breeding efforts have been implemented at several locations in Texas in an attempt to increase their numbers and save them from extinction. In the mid-1960s, the World Wildlife Fund established the Attwater's Prairie Chicken National Wildlife Refuge near Eagle Lake, Texas. The Nature Conservancy of Texas established the Texas City Prairie Preserve for the Attwater's prairie chicken in 1995. Captive breeding programs have been established at Fossil Rim Wildlife Center, the Small-Upland bird Research Facility (SURF) at Texas A&M University, the Houston, Abilene, San Antonio and Caldwell Zoos and Sea World of Texas. In spite of these efforts, the numbers continue to dwindle and the final demise of the Attwater's also may be disease.

REV BACKGROUND

General

Reticuloendotheliosis virus (REV) is a retrovirus exhibiting a type C morphology which is characterized by a symmetrically placed spherical inner core and viral assembly at the plasma membrane of the host cell. It is classified in the gammaretrovirus genera, previously considered the mammalian type C viruses, and includes such viruses as murine leukemia virus (MuLV) and feline leukemia virus (FeLV). The simple genome is approximately 9.0 kilobases (Coffin, 1982) and encodes 4 genes - *gag*, protease (*pro*), polymerase (*pol*), and envelope (*env*). A defective REV is also known that carries an oncogene that replaces some of the genome rendering it oncogenic but replication incompetent (Coffin, 1982). The RNA genome of the defective REV is approximately 5.7 kb and can be packaged into infectious particles in the presence of competent helper REV.

The coding region of the RNA genome is flanked on the 5' and 3' ends by 2 unique regions designated U5 and U3, respectively. These unique regions are flanked on the outside by a repeated region (R), present on each end of the genome. The mechanism of reverse transcription elicited by the reverse transcriptase enzyme results in a double stranded DNA genome consisting of the coding region flanked by two identical long terminal repeats (LTRs). The LTRs, present on each side of the coding region,

consist of the sequence U3-R-U5. The entire double stranded DNA genome is integrated into the host cell genome by the integrase enzyme.

The infectious viral particle, capable of infecting and replicating within a host cell, has a diameter of approximately 100 nanometers. It consists of two copies of the RNA genome encased by the inner core proteins. These inner core proteins which include the matrix, capsid and nucleocapsid proteins are encoded by the gag sequence. The RNA genome is associated with nucleocapsid protein and the polymerase proteins, reverse transcriptase and integrase, which comprise the nucleocapsid core of the virus. The nucleocapsid protein is surrounded by the matrix protein, which is surrounded by the virion envelope. The envelope is a lipid bilayer derived from the host cell's plasma membrane and contains the viral glycoproteins that are post translationally cleaved into transmembrane and surface products. The transmembrane (TM) spans the bilayer once and is bound to the surface (SU) subunit, which exists entirely outside of the virion complex. This surface protein is responsible for binding to host cell surface receptors, initiating infection.

Host range and strain classification

REV represents several closely related viruses that were originally isolated from various avian species. REV strain T was originally isolated from turkeys (Robinson and Twiehaus, 1974). Strain T carries an oncogene, v-rel, and is defective for replication as a portion of the genome is replaced by the oncogene (Chen et al., 1981; Hoelzer,

Franklin, and Bose, 1979). A nondefective helper virus, REV-A, is necessary for replication of the T strain, but also exists in the absence of the defective virus (Hoelzer, Franklin, and Bose, 1979). Other RE viruses include chick syncytial virus (CSV) (Cook, 1969), isolated from chickens, as well as spleen necrosis virus (SNV) (Trager, 1959) and duck infectious anemia virus (DIAV) (Ludford, Purchase, and Cox, 1972), both isolated from ducks. RE viruses have also been isolated from various other avian species including pheasants, quail, geese, peafowl, and prairie chickens (Dren, 1983; Dren, 1988a; Drew et al., 1998; Miller et al., 1998; Schat et al., 1976).

REV strain differences have been analyzed based on several different criteria. Purchase and Witter (1975) described variations in pathogenicity among the viruses in different hosts, including turkeys, chickens, and ducks. Strain T displayed the highest level of pathogenicity among all viruses in both the turkey and chicken hosts, whereas SNV elicited a disease of comparable severity in ducks. DIAV exhibited a moderate pathogenicity in ducks as compared to SNV, but resulted in a milder disease in chickens. CSV infection resulted in the lowest level of pathogenicity among all viruses, eliciting a disease of minimal severity in both chickens and ducks comparable to that produced by DIAV in ducks alone. Despite these differences, severity of disease caused by the different isolates is not used as a basis for strain classification.

Comparisons of neutralizing antibody titers have established the RE viruses to be of one serotype (Chen et al., 1987). Cross reactivity of neutralizing antibodies with various strains of viral isolates from different hosts routinely neutralized virus at low relative titers (Chen et al., 1987; Witter, Purchase, and Burgoyne, 1970). However,

Chen et al. (1987) describe variations in cross-reacting neutralizing antibody titers that distinguish 3 different subtypes. Neutralizing antibody titers within each group were lower than those among the different subtypes. Viruses in group 1 consisted mostly of strain T isolates from turkeys. The second group included both SNV and DIAV as well as another duck isolate and several turkey isolates. Group 3 included CSV as well as several other isolates from chicken. Two pheasant isolates also associated with group 3 viruses. Groups 1 and 2 showed the greatest disparity with the highest antibody titers required for neutralization among all groups. The association of DIAV and SNV within the same subtype, indicates these two viruses share the closest relationship among all 4 of the prototypes. The greatest difference antigenically among the prototypes is seen between either SNV and DIAV with the REV strain T. A lesser degree of difference is seen between the subtype including CSV and the other two.

Monoclonal antibody reactivity among the viruses supports the antigenic distinction of these three groups. Chen et al. (1987) describe an epitope present exclusively in group 1 viruses and another epitope shared by only groups 1 and 3. The exclusion of group 2 viruses for both of these epitopes confirms the differences among the 3 groups. Cui et al. (1986) also have identified epitopes present on REV-T that were not present in CSV. Sequence identities among viruses from the different subtypes, as well as epitope mapping, have not been described to identify specific regions of variability in these viruses.

Horizontal transmission

Understanding transmission of REV is critical in designing methods to prevent spread of the virus. Although it is not completely understood how the virus is spread, direct contact of infected birds with uninfected birds can result in transmission (Larose and Sevoian, 1965; Motha, 1984; Paul et al., 1977). Separation of birds with wire mesh has proven to prevent the spread of virus among birds (Bagust, 1981) lending evidence to the possibility that aerosol contact is insufficient for transmitting the virus. More likely, contact with feces or bodily fluids is required for transmission to occur as REV has been detected in fecal samples, cloacal swabs, and bodily fluids (Bagust, 1981; Bagust, 1979; Peterson and Levine, 1971; Witter, Smith, and Crittenden, 1981). Despite the instability and rather quick degradation of REV outside the host (Baxter-Gabbard et al., 1971), viable virus also has been detected in litter samples (Witter and Johnson, 1985), most likely surviving in feces. Removal of infected birds from the flock along with litter disposal and disinfection are critical in limiting the spread of disease within populations of housed birds.

Although physical contact, along with the presence of virus in feces, can account for transmission of REV within a flock, the transmission of REV in previously uninfected flocks is more difficult to explain. Mosquitoes present a possible mechanism to transport REV into flocks from infected wildlife in surrounding areas. Seroconversion of both chickens (Motha, Egerton, and Sweeney, 1984) and captive prairie chicken flocks (personal observations) during summer months is consistent with

the possibility that mosquitoes are involved in transmission as mosquito numbers tend to increase at this same time. REV has been isolated from two species of mosquitoes, *Triatoma infestans* and *Ornithodoros moubata* (Thompson, Fischer, and Luecke, 1968; Thompson, Fischer, and Luecke, 1971), following feeding on infected birds, although virus was unable to be cultured following isolation . Additionally, Motha, Egerton, and Sweeney (1984) were able to demonstrate experimental mechanical transmission with mosquitoes. The implication of mosquitoes involved in the transmission of REV raises several management concerns for captive populations in efforts to prevent the spread of virus by eliminating contact of the birds with mosquitoes.

Poxvirus presents another interesting possibility for REV transmission. Incorporation of the REV genome within poxvirus DNA has been previously described (Hertig et al., 1997). As poxvirus is a relatively stable virus capable of surviving outside the host, this association with REV presents an ideal mechanism for transmission of the more unstable REV. Incidence of REV within captive prairie chicken flocks has coincided with incidences of pox lesions occurring at the same time (personal observations).

Vertical transmission

Vertical transmission is typically associated with tolerantly infected birds having persistent viremia resulting in shedding of virus to produce infected progeny. Vertical transmission rates in tolerantly infected birds have been demonstrated to be as low as 8%

in turkeys (McDougall, 1980) and 3-5 % in chickens (Bagust, 1981; Witter, Smith, and Crittenden, 1981). However, vertical transmission rates of up to 50% in chickens (Motha and Egerton, 1987) and 87% in ducks (Motha, 1984) have been reported. Vertical transmission of REV in non-tolerantly infected females is uncommon (Witter, 2003).

PATHOGENESIS OF REV

Infection and immunity

Tolerant infections are defined by the lack of an antibody response mounted following challenge with the virus. Non-tolerant infections result from an antibody response and a transient viremia. Tolerant infection is commonly seen in birds infected prior to hatch by either *in-ovo* inoculation or vertical transmission (Bagust, 1981; Ianconescu, 1978; Witter, Smith, and Crittenden, 1981). Chickens infected at hatch or later typically have a greater capability of mounting an antibody response to REV and rarely incur a persistent infection (Bagust, 1979; Peterson and Levine, 1971; Purchase et al., 1973; Witter and Johnson, 1985; Witter et al., 1979; Yuasa, Yoshida, and Taniguchi, 1976). Non tolerantly infected birds typically mount an antibody response within 2-20 weeks following exposure by either inoculation or physical contact with infected birds (Bulow, 1977; Ianconescu, 1978; Larose and Sevoian, 1965; McDougall, 1980; Motha, 1982). Although less common, persistent infections have been described in some

chickens following inoculation with REV at hatch (Bagust, 1979; McDougall, 1980; Witter, Smith, and Crittenden, 1981). Lack of persistent infection established in birds infected after hatch is consistent with the rarity in clinical disease seen following infection of older chickens (Peterson and Levine, 1971; Purchase et al., 1973; Witter and Johnson, 1985; Yuasa, Yoshida, and Taniguchi, 1976). However, Witter et al. (1981) reported a higher incidence of neoplasia in non-tolerantly infected chickens as compared with birds with persistent viremia. Turkeys appear to be susceptible to disease following infection after hatch as persistent infections do occur (McDougall, 1980; Witter and Salter, 1989) and lymphomas have developed in birds exposed to virus by physical contact with other infected birds (McDougall, 1980; McDougall, 1981; Paul et al., 1977).

Runting disease

The runting disease caused by REV is characterized by an array of non neoplastic clinical symptoms resulting from infection (Witter, 2003). Weight depression among infected chickens and ducks has been observed (Motha, 1987; Mussman and Twiehaus, 1971; Purchase et al., 1973). Chicks affected by weight depression typically do not overcome the stunting in growth (Motha, 1987). Coincident with weight depression, Mussman and Twiehaus (1971) also reported atrophy of both the bursa and thymus in chicks infected with REV. Abnormal feather development, termed “Nakanuke”, has also been reported in infected chicks, resulting from necrosis of feather forming cells

(Koyama et al., 1976; Tajima, Nunoya, and Otaki, 1977). Gross peripheral lesions resembling those caused by Marek's disease virus infection have been described by Witter et. al. (1981). These lesions were the result of infiltration of lymphocytes and plasma cells. However, unlike Marek's disease virus infection, gross nerve lesions did not result in lameness or paralysis in affected birds.

The runting disease caused by REV infection has also been attributed to immunosuppression. Depressed antibody responses to secondary infections with Marek's disease virus, Newcastle disease virus, turkey herpes virus, and brucella abortus have all been reported (Bulow, 1977; Ianconescu, 1978; Kawamura et al., 1976; Witter et al., 1979; Yoshida et al., 1981). Inhibited allograft rejection attributed to REV infection has also been noted (Walker et al., 1983). Additionally, spleen cells from infected chickens have shown a decreased ability to respond to phytohemagglutinin (Carpenter, Bose, and Rubin, 1977; Scofield and Bose, 1978)(37, 198). Ferro (2001) described a decrease in $CD4^+$ T cells resulting from REV infection in greater and Attwater's prairie chickens that was not unlike the disease caused by HIV infection in humans.

Neoplasia in chickens

Much of the research on neoplasias induced by REV has been conducted in chickens although neoplastic lesions have also been described in other avian species. Among all neoplastic lesions found in all species, the liver and spleen were most

commonly reported to be affected (Witter, 2003). Neoplasias induced by REV infection can arise as either acute or chronic manifestations. These two types of neoplasias differ greatly in cause of tumor development, incubation times and tissue types affected (Witter, 2003). Chronic lymphomas in chickens can be subdivided into two groups consisting of bursal and nonbursal lymphomas.

Bursal lymphomas in chickens arise as a result of REV proviral integration adjacent to the cellular oncogene c-myc (Fujita, 1984; Ridgway et al., 1985; Swift et al., 1985). Expression of the c-myc epitope as a result of this integration was shown to be increased 50-100 fold above normal cells (Hayward, 1981). Bursal lymphomas are characterized by a high incidence of neoplastic lesions found in the bursa with lesions in the liver also being common (Witter and Crittenden, 1979; Witter, Smith, and Crittenden, 1981). Lesions are typically indistinguishable from those induced by avian leukosis virus (ALV) (Witter and Crittenden, 1979). Witter et. al (1981) report 96% of birds with lymphoma with bursal lesions with neoplasia being found in the liver in 92% of affected birds. Neoplastic lesions were also reported in the spleen, kidney, gonads, intestine, proventriculus, brain, nerves, thymus, and lungs (Grimes, 1979; Witter and Crittenden, 1979; Witter, Smith, and Crittenden, 1981). Neoplastic lesions developed between 17-43 weeks after infection with REV (Witter, Smith, and Crittenden, 1981). Involvement of the bursa has been further confirmed by the absence of tumors in bursectomized birds as compared with significant neoplasia in birds with intact bursa. Nazerian et. al. (1982) report the involvement of B cells in these lymphomas based on

antibody labeling of B cells in tumors induced by CSV. T cells were not present in any of the tumors examined.

Non-bursal lymphomas have been reported in chickens experimentally infected with CSV and SNV strains (Witter, Sharma, and Fadly, 1986). Tumors were found in the heart, thymus, liver, and spleen, but not in the bursa. Bursal atrophy was noted in affected birds. Lymphoma development was noted between 40-50 days post infection with a mean time until death of about 16 weeks – notably shorter than the latency of bursal lymphomas. Analysis of tumor cells revealed the presence of T cell markers, identified by the common T cell marker CD3, whereas B cell markers were not present (Cooper, 1991). The majority of the cells expressed the CD8 antigen, although some expressed CD4 or both CD4 and CD8. Like bursal lymphomas, non-bursal lymphomas also develop as a result of proviral integration near the c-myc oncogene in the host genome. Two to four copies of proviral DNA were typically present in these tumor cells, one of which was integrated close to the c-myc (Isfort, Witter, and Kung, 1987). However, c-myc expression of this integration was markedly lower, only 6-18 fold above that of normal cells. Non-bursal lymphomas have not been reported in birds naturally infected with REV (Witter, 2003).

Acute reticulum cell neoplasia is caused by infection of the replication deficient REV-T which carries the v-rel oncogene along with coinfection of a replication competent REV-A. Infiltrating cells are usually composed of mononuclear reticuloendothelial cells or primitive mesenchymal cells (Robinson and Twiehaus, 1974; Sevoian, 1964b; Theilen, Zeigel, and Twiehaus, 1966). Lesions typically involve

enlarged livers and spleens as a result of infiltration by neoplastic cells (Sevoian, 1964a; Theilen, Zeigel, and Twiehaus, 1966). Other organs commonly affected include the heart, kidney, pancreas, and gonads. Incubation time following infection to the development of neoplasia is strikingly shorter than in chronic lymphomas, with manifestations of disease arising within 3 days to 3 weeks following infection (Sevoian, 1964a). This disease is associated with high mortality (Sevoian, 1964a).

Neoplasia in other avian species

Although many studies have described neoplasias in chickens, neoplasia in various other avian species has also been observed. REV was originally isolated from turkeys (Robinson and Twiehaus, 1974). Experimental infections of turkeys have shown the most common organs affected by REV induced neoplasias to be the liver and spleen (McDougall, 1978; Paul et al., 1977). The intestines were also commonly affected. Other organs and tissue types developing neoplasia include the pancreas, heart, lungs, kidneys, peripheral nerves, gonads, thymus, and bone marrow esophagus (McDougall, 1978; Paul et al., 1977). Hayes et. al. (1992) described neoplastic lesions in the esophagus of wild turkeys infected with REV. Incubation periods of 8 to 11 weeks (Paul et al., 1977) and 15 weeks (McDougall, 1978) have been reported with mortality greater than 20%.

Neoplastic lesions in ducks have been observed in the liver, spleen, intestines, skeletal muscle, pancreas, lung, thymus, kidney, heart, brain, and peripheral nerves

(Grimes and Purchase, 1973; Li et al., 1983; Motha, 1984). Incubation time of the virus from infection to development of lymphomas has been observed from as early as 4 weeks to 30 weeks (Grimes and Purchase, 1973; Li et al., 1983; Motha, 1984; Paul, Werdin, and Pomeroy, 1978; Perk, 1981). Studies in geese have described chronic lymphomas developing between 20 and 30 weeks after infection (Dren, 1988a). Similar neoplastic lesions as a result of REV infection have also been found in quail (Schat et al., 1976), peafowl (Miller et al., 1998), pheasants (Dren, 1983), partridges (Trampel, Pepper, and Witter, 2002) and prairie chickens (Drew et al., 1998). Neoplastic lesions in the organs of peafowl, pheasants, and prairie chickens have occasionally been accompanied by lesions on the head, in the mouth and on the legs and feet (Dren, 1983; Drew et al., 1998; Miller et al., 1998). REV has also been isolated from similar lesions observed in domestic turkeys (Linares, unpublished data)

DETECTION

A method of REV detection by PCR amplification of LTR sequences and Southern blot has been previously described (Aly, 1993). As this method does not detect genes encoding structural proteins, the potential exists for detecting LTR sequences present in the absence of whole virus genome sequences. Furthermore, the limit of sensitivity of this PCR assay is 600 copies of the LTR per reaction with the additional labor intensive step of Southern blotting (Aly, 1993). Routine assays for REV detection typically use only the PCR amplification of the LTR described by Aly et al. (1993) without the final

step of Southern blotting, thus significantly decreasing the sensitivity of the test. As early detection is necessary for the removal of infected birds to limit the spread of infection in captive populations, a more sensitive and convenient PCR detection method aimed at protein encoding genome regions is preferred.

REV IN ATTWATER'S

Drew et. al (1998) reported the first documented cases of reticuloendotheliosis infection in the Attwater's prairie chicken. Two wild-caught greater prairie chickens developed multiple lesions on the face, legs, and feet. Following necropsy, all lesions on these birds were determined to be limited to the skin and consisted of lymphocytic-histiocytic proliferative and infiltrative lesions. Subsequent infections were discovered in both greater and Attwater's prairie chickens, which developed multifocal, neoplastic lesions that were more common in the liver and spleen. No external lesions were found in these birds. All lesions were consistent with disease caused by REV in other birds. Many of the prairie chickens that died or were euthanized tested positive for REV by PCR, but none of the infected Attwater's and less than 28% of the infected greater's tested positive for neutralizing antibody. This is consistent with chronic neoplasia and an immune tolerant infection seen in other avian species (Witter, 2003).

The original outbreak of REV at the Texas A&M University breeding facility has been followed by subsequent outbreaks at all of the captive breeding facilities in the state. Current procedures for handling these outbreaks have involved regular screening

of birds for REV by PCR as often as every two weeks and euthanasia of all birds testing positive. The result has been a significant loss of birds from the already depleted species. Management practices aimed at eliminating infection in the captive populations have been unsuccessful as positive birds are regularly identified. As the primary goal of these captive facilities is to increase the numbers of Attwater's prairie chickens, understanding and eliminating the threat caused by REV is critical to their efforts.

VACCINE

Although management practices can be utilized to minimize exposure of captive flocks to REV and remove infected birds to limit transmission within the flock, vaccination is a desired method for protection against REV infection. Attenuated viruses are commonly used for vaccination of other viruses, however, they are not an option for use in an endangered species infected by a retrovirus responsible for immunosuppression and neoplasia. Proviral insertion into the host genome results in vertical transfer to dividing daughter cells, assuring the presence of the virus within the host for the life of the host. The potential for an attenuated strain of the virus to revert to a more pathogenic strain could result in more harm than benefit if uninfected birds are vaccinated in this manner.

DNA vaccines provide a suitable alternative to attenuated viruses. Due to the fact that only a portion of the genes are inserted into expression vectors, DNA vaccines are unable to revert to a pathogenic strain (Orson, 2000). Additionally, there is no chance of

the spread of an inadvertent infection (Johnston, 1997). The relatively high mutation rate among some retroviruses is also more easily accounted for as DNA vaccines can be manipulated with ease to include new or modified sequences (Johnston, 1997). DNA vaccines have also been shown to provide long-lived immunity, unlike component vaccines which may require multiple inoculations (Yankauckas, 1993).

Much of the original DNA vaccine research has been described in mice (Donnelly, 1997). Intramuscular immunization of mice with DNA plasmids encoding the hemagglutinin (HA) and neuraminidase (NA) genes of influenza has been shown to generate long-lived antibody responses (Justewicz, 1995; Raz, 1994; Yankauckas, 1993). $CD8^+$ cytotoxic T cell responses have also been demonstrated with intramuscular injections of the influenza nucleoprotein (NP) DNA (Ulmer, 1994) and have been shown to persist for more than 2 years (Donnelly, 1996; Yankauckas, 1993). Additionally, intramuscular immunization with plasmids encoding influenza HA, influenza NP, human immunodeficiency virus (HIV) env, HIV gag and HIV rev have resulted in proliferation of $CD4^+$ T helper cells which secreted the cytokines interferon gamma (IFN-γ) and IL-2, indicative of a Th-1 like response (Shiver, 1995). Th-2 like responses have been seen following epidermal vaccinations with a gene gun (Fuller, 1994).

DNA vaccines have also been used in avian species to provide protection against viruses and bacteria (Oshop, 2002). Early work on DNA vaccines was focused on avian influenza in chickens. DNA immunization of chickens against avian influenza has been shown to produce significant antibody and cell-mediated immune responses which resulted in protection (Fynan, 1993). Other avian viruses including Newcastle disease

virus (NDV)(Sakaguchi, 1996), infectious bursal disease virus (IBDV) (Chang, 2001; Fodor, 1999), and infectious bronchitis virus (IBV) (Kapczynski, 2001; Seo, 1997) have also been studied with regards to DNA immunization and have been used to generate partial and complete protection. Several routes of delivery of plasmid DNA have been investigated, however, the intramuscular (i.m.) route has been shown to be the most effective and is still most commonly used (Oshop, 2002). A broad range of DNA concentrations have been used from 0.25 μg/bird (Kodihalli, 1997) to 750 μg/bird (Triyatni, 1998) and dose responses have been reported (Suarez, 2000). However, a limited amount of DNA at 100 μg/bird injected i.m. was shown to give the best response without diminishing the antibody response (Suarez, 2000).

OBJECTIVES

The specific objectives of this research project are as follows:

- Develop a method of flow cytometry in avian peripheral blood mononuclear cells capable of excluding thrombocytes from lymphocyte populations for greater accuracy in monitoring lymphocyte subpopulations.
- Develop a set of reagents to identify prairie chicken and REV specific antigens for use in further examination of REV infection in prairie chickens.
- Conduct a phylogenetic analysis of REV prototypes and prairie chicken isolates to determine the genetic relationship and variability among the viruses.
- Identify the target cell of REV infection.

- Develop a sensitive method of REV detection for early identification and culling of infected birds in captive populations.
- Observe the pathogenesis of REV in the experimental infection of hybrid greater/Attwater's prairie chickens and establish a minimum infectious dose.
- Develop a DNA vaccine to be used in potential therapeutic and/or prophylactic protection.
- Test a DNA vaccine on infected prairie chickens for its capability to reduce detectable viral levels and perhaps minimize vertical transmission.

CHAPTER II

PHYLOGENETIC ANALYSES INDICATE LITTLE VARIATION AMONG RETICULOENDOTHELIOSIS VIRUSES INFECTING AVIAN SPECIES, INCLUDING THE ENDANGERED ATTWATER'S PRAIRIE CHICKEN*

Reticuloendotheliosis virus infection, which typically causes systemic lymphomas and high mortality in the endangered Attwater's prairie chicken, has been described as a major obstacle in repopulation efforts of captive breeding facilities in Texas. Although antigenic relationships among reticuloendotheliosis virus (REV) strains have been previously determined, phylogenetic relationships have not been reported. The pol and env of REV proviral DNA from prairie chickens (PC-R92 and PC-2404), from poxvirus lesions in domestic chickens, the prototype poultry derived REV-A and chick syncytial virus (CSV), and duck derived spleen necrosis virus (SNV) were PCR amplified and sequenced. The 5,032 bp, that included the pol and most of env genes, of the PC-R92 and REV-A were 98% identical, and nucleotide sequence identities of smaller regions within the pol and env from REV strains examined ranged from 95 to 99% and 93 to 99%, respectively. The putative amino acid sequences were 97 to 99% identical in the polymerase and 90 to 98% in the envelope. Phylogenetic analyses of the nucleotide and amino acid sequences indicated the closest relationship among the recent

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pox-associated chicken isolates, the prairie chicken isolates and the prototype CSV while only the SNV appeared to be distinctly divergent. While the origin of the naturally occurring viruses is not known, the fowl poxvirus may be a critical component of transmission of these ubiquitous oncogenic viruses.

INTRODUCTION

Reticuloendotheliosis virus (REV) is an avian gamma retrovirus with an approximately 9.0 kb genome that encodes the gag, pol and env ORFs. Clinical manifestations of infection result in immunosuppression, runting disease, and lymphomas (Witter, 2003). REV strains have a wide avian host range that include chickens, turkeys, ducks, geese, pheasants, peafowl and prairie chickens, a type of grouse (Chen et al., 1987; Dren, 1988a; Drew et al., 1998; Miller et al., 1998). Established laboratory strains of REV include REV-A, its tumor forming counterpart, REV-T, chick syncytial virus (CSV), spleen necrosis virus (SNV), and duck infectious anemia virus (DIAV) (Cook, 1969; Hoelzer, Franklin, and Bose, 1979; Ludford, Purchase, and Cox, 1972; Trager, 1959). Both SNV and DIAV were isolated from ducks.

Drew et al. (1998) reported the first incidence of prairie chicken REV in 7 greater (*Tympanachus cupido pinnatus*) and 2 endangered Attwater's prairie chickens (*Tympanachus cupido attwateri*). Lesions on the face, legs, and feet of the infected birds were similar to poxvirus lesions previously described in other species (Dren, 1983;

Miller et al., 1998). External lesions were not observed in additional REV infected birds, but neoplastic lesions were typically found in livers and spleens, consistent with REV infections seen in chickens and turkeys (Dren et al., 1988b; Hayes, Langheinrich, and Witter, 1992; Ratnamohan et al., 1980; Witter and Crittenden, 1979; Witter, Smith, and Crittenden, 1981). Subsequent to the initial outbreak of REV in prairie chickens, REV infections have reoccurred at 6 other captive breeding facilities for endangered Attwater's prairie chicken in Texas (Bohls, unpublished data). Losses of Attwater's prairie chicken associated with REV infection, that typically results in lymphomas, have threatened breeding efforts for repopulation. Although critical in devising strategies to prevent further losses, the source of REV infecting individuals within a flock and involved in flock to flock transmission is not known. In the current study, nucleotide sequences and putative amino acid sequences of the env and pol from REV prototype strains and REV isolated from Attwater's prairie chicken and domestic chickens in Texas were determined and compared. Nucleotide and amino acid analyses indicated that although all genome regions examined were greater than 93% identical, the Texas isolates and the CSV are more closely related.

MATERIALS AND METHODS

Virus

Sources of the viruses are summarized in Table 1. Virus isolates representing three of the known groups of REV strains, including REV-A, CSV and SNV were

Table 1. Source of viruses.

Virus	Source	GenBank Accession #
Nondefective REV-A	Chicken (Hoelzer, Franklin, and Bose, 1979)	DQ237900
Spleen necrosis virus (SNV)	Duck (Cook, 1969; Trager, 1959)	DQ237902, DQ237903
Chick syncytial virus (CSV)	Chicken (Cook, 1969)	DQ237904, DQ237905
PC-R92	Attwater's Prairie chicken (Texas)	DQ237901
TX-01	Chicken (Texas	DQ237906, DQ237907
TX-04	Chicken (Texas)	DQ237908, DQ237909
PC-2404	Attwater's Prairie chicken (Texas)	DQ237910, DQ237911

generous gifts from Dr. Aly Fadly at USDA, Michigan State University, East Lansing, MI. The reticuloendotheliosis virus strain, PC-R92, was isolated from a naturally infected Attwater's prairie chicken following the initial outbreak of REV in the Attwater's flock at Texas A&M University in 1994 (Drew et al, 1998). Another prairie chicken isolate, PC-2404 was isolated from an infected prairie chicken in Texas in 2003. The TX-01 and TX-04 strains were isolated from confirmed pox lesions of naturally infected backyard chickens. Lesions isolated from the trachea and sinus of TX-01 in 2004 and the trachea, sinus and cloaca of TX-04 in 2003 were grown on embryonated chicken egg chorioallantoic membranes (CAM's) producing histopathological lesions that confirmed pox infection. CAM's from TX-01 and TX-04 and all other virus isolates were cultured on DF-1 cells (American Type Culture Collection) for 3 days in Dulbeco's minimal essential media (DMEM) with 2% FBS in 25 cm^2 flasks. Supernatants were removed and frozen at -80°C. Infected cells from each flask were harvested and genomic DNA was collected using the GenElute Mammalian genomic DNA Miniprep kit (Sigma, St. Louis, MO). PCR amplification of REV specific sequences in the genomic DNA of DF-1 cells confirmed the presence of the virus.

PCR

Primers used to amplify the pol and env genes of all viruses were initially designed based on an unpublished spleen necrosis virus sequence (Table 2). Primer pairs within the polymerase amplified 2 overlapping fragments of 742 and 580 base pairs that

Table 2. Primers used for PCR amplification and sequencing.

Gene[a]	Forward 5' – 3'	Reverse 5' – 3'
Pol (2456, 2984)	GCCCCAGTATTGGTAGAGGAATTACA	TGGGGGCTTGTGTGGATGC
Pol (2883, 3624)[b]	CGCCGATACAGAATGTTAC	TGGGCCTTTTTCCCTGAAAC
Pol (3559, 4138)[b]	TGCCACCCGAGACTTACTCA	CTGCCCGAAGGTAAGTTTAGAG
Pol (3874, 4428)	AGGCGTTCCAGAGTTTGAAGC	CTGCCGTCAGTGAACAAGGTG
Pol (4367, 4942)	CGCCCCGACCTGACGGATCAGC	CCCCCTTAGTCGATGTCCTAGAG
Pol (4797, 5306)	ACCGACGGGCAGATGAGGTG	CCCAGCCCGAGAATGTGTCTAC
Pol (5201, 5779)	GGTGCGGCTCCAGGGGAACACT	TGCCCGAGCCAGAGACCTAGTG
Pol (5684-6094)	CCCCGTGTGGGATGTGACAAG	ACCCCCACCAAGCCACAAGGAGAA
Pol (5825-6269)	CCCCACTGTTCCAACCTGGTGATCT	CCCCCTCCCCAGCTCCAACTCC
Env (6157, 6674)[b]	GGGGGATATGTCTCCTCCATACCTA	CGGGGCAGGGCTAAAGGGTGATACTGT
Env (6565, 7077)[b]	GGCGGCGGTCCCACTGACAT	TGCCGTGTAGGCCATGTTGTTCC
Env (6937, 7487)	CCCGTAGGGTATGTCCATTTTACT	CCCGACTTGTTAGCGTAAAAA

[a]Gene and nucleotide region amplified

[b]Primers used for amplification in all viral isolates. Undesignated primer pairs were used only for sequencing of R92 and REV-A in nucleotide identity comparisons.

spanned a total of 1256 base pairs beginning approximately 275 base pairs downstream of the pro/pol start codon. Primer pairs within the envelope amplified two overlapping fragments of 518 and 513 that spanned a total of 921 base pairs beginning 150 base pairs down stream of the env start codon. Additional primer pairs were used to amplify a 5032 base pair region extending from the polymerase through the envelope (Fig. 1). Two µl of genomic DNA template were added to a 50 µl reaction containing 36.75 µl water, 5 µl 10X Mg free reaction buffer (Promega, Madison, WI), 3 µl 10 mM $MgCl_2$, 1 µl 10 mM dNTP mix, 1 µl (10 pmol) of forward primer, 1 µl (10 pmol) reverse primer and 0.25 µl Taq polymerase (Promega, Madison, WI). Samples were incubated at 95º C for 5 minutes, then cycled at 95º C for 30 sec., 55º C for 30 sec and 72º C for 2 min for 40 cycles. Samples were incubated at 72º C for 7 min, before storing at 4º C. PCR products were purified with a GenElute PCR clean-up kit (Sigma, St. Louis, MO).

Sequencing

PCR products were adjusted to 5 ng /µl before adding 2 µl to a 6 µl reaction also consisting of 2 µl Big Dye (Perkin Elmer, Norwalk, CT), 1 µl water and 1 µl (10 pmol) primer. Thermal cycling steps for 60 cycles were 96º C for 10 sec, 50º C for 5 sec and 60º C for 4 minutes. Sequencing reactions were purified using Bio Max spin columns (Bio Rad, Hercules, CA) and dried in a speed vacuum. Samples were sent to the Gene Technologies core facility for DNA sequencing (Department of Biology, Texas A&M University). Nucleic acid and amino acid sequences were aligned using ClustalW

software (European Bioinformatics Institute). Phylogenetic trees were constructed with the use of MEGA3 software and bootstrap tests of phylogeny were used to construct neighbor joining phylogenetic trees under the phylogeny menu.

RESULTS

Nucleotide sequence comparisons indicate a close relationship among REV strains

Prototype viruses REV-A, CSV and SNV were available for sequencing as well as two Texas prairie chicken isolates, PC-R92 (isolated in 1994) and PC-2404 (isolated in 2003). REV isolates from poxvirus lesions on two backyard chickens in Texas, TX-01 (isolated in 2004) and TX-04 (isolated in 2003), also were available. Initially, a total of 5031 bases of both PC-R92 and REV-A was sequenced and compared (Fig. 1). Overall, the sequenced pol and env regions of the two genomes were 98% identical.

In order to determine divergence within the REV group of viruses, polymerase and envelope sequences from genomes of all other isolates, also were determined and compared with the R92 and REV-A strains. Percent identities of the regions sequenced were between 95 to 99% for the pol and 93 to 99% identical for the env sequences (Table 3). A phylogenetic tree was generated from a fusion of pol and env nucleic acid sequences. The larger data set resulting from the concatenated sequences provided more phylogenetic informative positions than individual sequences for tree construction (Weaver, 2004). Analysis of the resulting phylogenetic tree indicated that the Texas

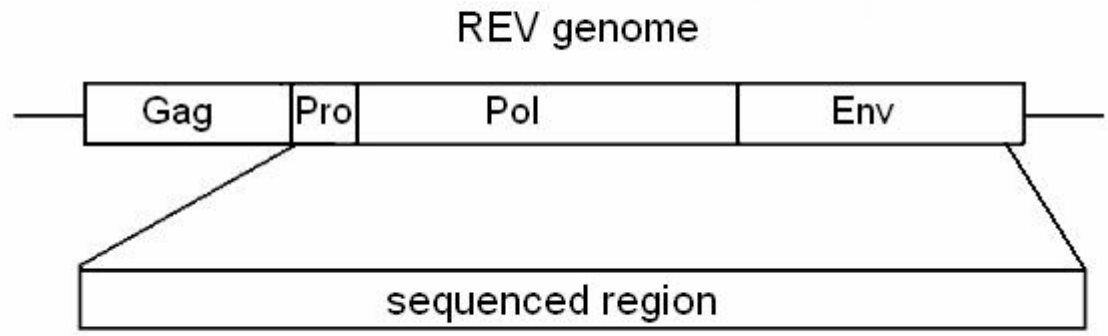

Figure 1. Genomic map of sequenced region of REV-A and the prairie chicken isolate R92. Identity between the two viruses was found to be 98%.

Table 3. Percentage nucleic acid identity among the various isolates. Identity of the envelope gene is shown on the bottom left half of the table. Identity of the polymerase gene is shown on the top right half of the table.

	SNV	REV-A	CSV	PC-R92	TX-01	TX-04	PC-2404
SNV		95	96	96	97	97	96
REV-A	93		98	98	98	98	98
CSV	95	97		99	99	99	99
PC-R92	95	97	99		99	99	99
TX-01	95	97	99	99		99	99
TX-04	94	95	98	98	99		99
PC-2404	95	96	99	99	99	99	

isolates from both domestic chicken and prairie chicken clustered together with CSV and the SNV was the most divergent from the prairie chicken derived REV (Fig. 2).

Amino acid sequence comparisons

Translated amino acid sequences were compared among the polymerase and envelope genes of all strains (Table 4). Polymerase amino acid sequences were at least 97% identical among all the REV genomes examined and 99% identical among the genomes of the isolates from Texas domestic chicken and the prairie chicken.

With percent identity ranging from 90 to 98%, more variability was found in the amino acid sequence of the envelope than the polymerase. Alignments of both polymerase and envelope amino acid sequences, shown in Figure 3, did not identify any region with a concentration of variation. The few differences were scattered throughout the translated proteins. Comparisons of the envelope amino acids confirmed the close relationship of the domestic chicken and prairie chicken Texas isolates with the prototype CSV as shown in the phylogenetic tree (Fig 4.). Consistent with the nucleotide analyses and the polymerase amino acid analyses, the REV-A prototype envelope protein was somewhat more divergent, and the SNV amino acid sequences were most divergent.

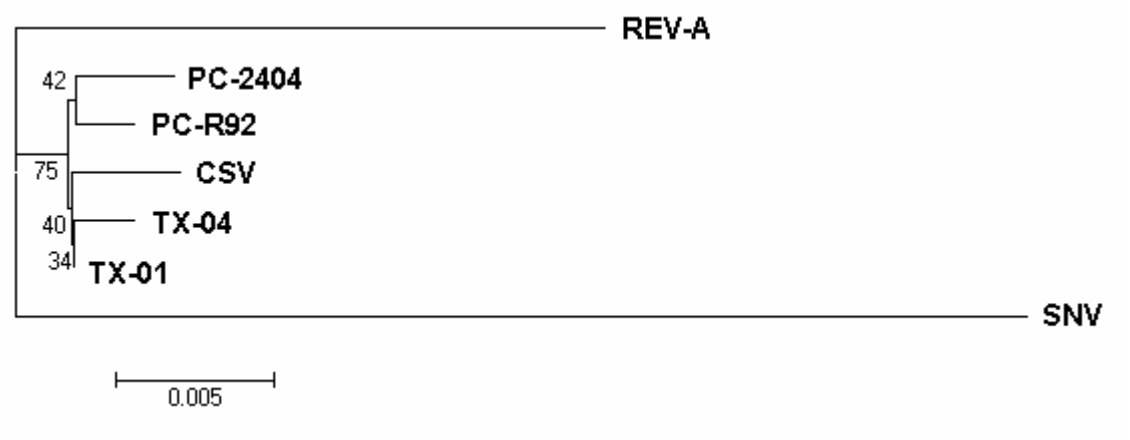

Figure 2. Phylogenetic tree of the constructed polymerase-envelope fusion nucleotide sequences. Numbers denote the percentage of 1000 bootstraps in which a certain branch occurred.

Table 4. Percentage amino acid identity among the various isolates. Identity of the envelope gene is shown on the bottom left half of the table. Identity of the polymerase gene is shown on the top right half of the table.

	SNV	REV-A	CSV	PC-R92	TX-01	TX-04	PC-2404
SNV		97	97	97	97	97	97
REV-A	92		98	98	98	98	98
CSV	90	94		99	99	98	98
PC-R92	93	95	97		99	99	99
TX-01	93	95	98	98		99	99
TX-04	91	91	95	95	96		97
PC-2404	92	92	96	97	97	97	

A.

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PC-R92     -PIQNVTLLE QWKREIPKVW AEINPPGLAS TQAPIHVQLL STALPVRVRQ
TX-01      -.......... ..........  .......... .......... ..........
TX-04      -.......... .......... .......... .......... ..........
PC-2404    ---------- ---....... .......... .......... .......I..
CSV        --........ .......... .......... .......... ..........
REV-A      -.......... .......... .......... .......... ..........
SNV        R......... .......... .......... .......... ..........

PC-R92     YPITLEAKRS LRETIRKFRA AGILRPVHSP WNTPLLPVRK SGTSEYRMVQ
TX-01      .......... .......... .......... .......... ..........
TX-04      .......... .......... .......... .......... ..........
PC-2404    .......... .......... .......... .......... ..........
CSV        .......... .......... .......... .......... ..........
REV-A      .......... .......... .......... .......... ..........
SNV        .......... .......... .......... .......... P.........

PC-R92     DLREVNKRVE TIHPTVPNPY TLLSLLPPDR IWYSVLDLKD AFFCIPLAPE
TX-1       .......... .......... .......... .......... ..........
TX-4       .......... .......... .......... .......... ..........
PC-2404    .......... .......... .......... .......... ..........
CSV        .......... .......... .......... .......... ..........
REV-A      .......... .......... .......... .......... ..........
SNV        .......... .......... .......... .......... ..........

PC-R92     SQLIFAFEWA DAEEGESGQL TWTRLPQGFK NSPTLFDEAL NRDLQGFRLD
TX-01      .......... .......... .......... .......... ..........
TX-04      .......... .......... .......... .......... ..........
PC-2404    .......... .......... .......... .......... ..........
CSV        .......... .......... .......... .......... ..........
REV-A      .......... .......... .......... .......... ..........
SNV        .........T .......... .......... .......... ..........

PC-R92     HPFVSLLQYV DDLLIAADTQ AACLSATRDL LMTLAELGYR VSGKKAQLCQ
TX-01      .......... .......... .......... .......... ..........
TX-04      .......... .......... .......... .......... ..........
PC-2404    .......... .......... .......... .......... ..........
CSV        .......... .......... .......... .......... ..........
REV-A      ..S....... .......... .......... .......... ..........
SNV        ..S....... .......... .......... .......... ..........

PC-R92     EEVTYLGFKI HKGSRTLSNS RTQAILQIPV PKTKRQVREF LGTIGYCRLW
TX-01      .......... .......... .......... .......... ..........
TX-04      .......... .......... .......... .......... ..........
PC-2404    .......... .......... .......... .......... ..........
CSV        .......... .......... .......... .......... ..........
REV-A      .......... .....S.... .......... .......... ..........
SNV        .......... .......... .......... .......... ..........

PC-R92     IPGFTELAQP LYAATRGGND PLVWGEKEEE AFQSLKLALT QPPALALPSL
TX-01      ....A..... .......... .......... .......... ..........
TX-04      ....A..... .......... .......... .......... ..........
PC-2404    ....A..... .......... .......... .......... ..........
CSV        ....A..... .......... .......... .......... ..........
REV-A      ....A..... .......... .......... .......... ..........
SNV        ....A..... .......... ..E....... .......... ..........
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Figure 3. Comparative alignment of putative polypeptide sequences. The polymerase sequence is represented in A and the envelope B. Gaps and unsequenced ends are represented by dashes. Perfectly-conserved amino acids relative to R92 are indicated by dots.

A.

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PC-R92     DKPFQLFVEE TGGAAKGVLT QALGPWKRPV AYLSKRLDPV AAGWPRCLRA
TX-01      .......... .......... .......... .......... ..........
TX-04      .......... .......... .......... .......... ..........
TX-2404    .......... .......... .......... .......... ..........
CSV        .......... .......... .......... .......... ..........
REV-A      .......... .S........ .......... .......... ..........
SNV        .......I.. .......... .......... .......... ..........

PC-R92     IAAAALLTRE ASKLTFGQ
TX-01      .......... ASKLTFGQ
TX-04      HC-------- --------
TX-2404    ......---- --------
CSV        .......... ........
REV-A      .......... ........
SNV        .......... .....W..
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Figure 3. Continued

B.

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PC-R92     GGYVFSIPTY YTYSLDCGSS TAYLTYGSGT GSWGWGGGFR QQWECVFKPK
TX-01      ....S..... ..N....... .......... .......... ..........
TX-04      .......... ..N....... .......... .......... ..........
PC-2404    .......... ..N....... .......... .......... ..........
CSV        .......... ..N....... .......... .......... ..........
REV-A      -........H ..N......F .........A .......... ..........
SNV        .......... .......... .......... .........K ..........

PC-R92     IIPSVQGQPG PCPSECLTIA TQMHSTCYEK AQECTLLGKT YFTAILQKTK
TX-01      .......... .......... .......... .......... ..........
TX-04      .......... .......... .......... .......... ..........
PC-2404    .......... .......... .......... .......... ..........
CSV        .......... .......... ..V....... .......... ..........
REV-A      ......E..R ..--...... .......... A......M... ..........
SNV        .......... .......Q.. .......... T......... ..........

PC-R92     LGSYEDGPNK LLQASCTGTI GKPVCWDPAA PVYVSDGGGP TDMIREESVR
TX-01      .......... .......... .......... .......... ..........
TX-04      .......... .......... .......... .......... ..........
PC-2404    .......... .......... .......... .......... ..........
CSV        .......... .......... .......... .......... ......D...
REV-A      .......... .........I. .........A. .......... ..........
SNV        .......... .I.......V .......... .......... ..........

PC-R92     ERLEEIIRHS YPSVQYHPLA LPRPRGVDLD PQTSDILEAT HQVLNATNPQ
TX-01      .......... .......... .......... .......... ..........
TX-04      .......... .......... .......... .......... ..........
PC-2404    .......... .......... .......... .......... ..........
CSV        .......... .......... .......... .......... ..........
REV-A      .......... .......... .......... .......... .........R
SNV        .......... .......... ...S...... .......... ..........

PC-R92     LAENCWLCMT LGTPIPAAIP ANGNVTLDGN CSLSLPFRVQ PTGSIDVNCY
TX-01      .......... .......... .......... .......... ..........
TX-04      .......... .......... ........... ......... .......K..
PC-2404    .......... .......... ........... ......... ..........
CSV        .......... .......... .......... .......... ...L......
REV-A      .......... .......... ...E...... .......... ....V.....
SNV        .......... .......... T......... .......... ..W.......

PC-R92     AGEADNRTGI PIGYVHFTNC TSIQEVSNET SHIRNLTRLC PPPGHVFVCG
TX-01      .......... .......... .......... .......... ..........
TX-04      .......... ........H. .......... .......S.. .....ES.--
PC-2404    .......... .......... .......... ..-------- ----------
CSV        .......... .......... .......... .......... ..........
REV-A      .......... .V........ .......... .......... ..........
SNV        .......... .V........ ......T... .QME...K.. ..........

PC-R92     NNMAYTALPN KW
TX-01      .......--- --
TX-04      ---------- --
PC-2404    ---------- --
CSV        .......--- --
REV-A      ....R----- --
SNV        .......--- --
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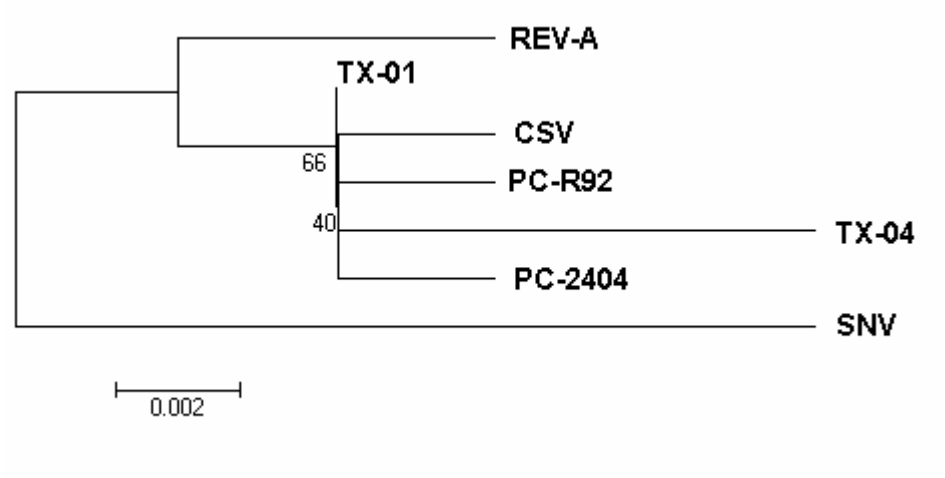
Figure 3. Continued

A.



B.

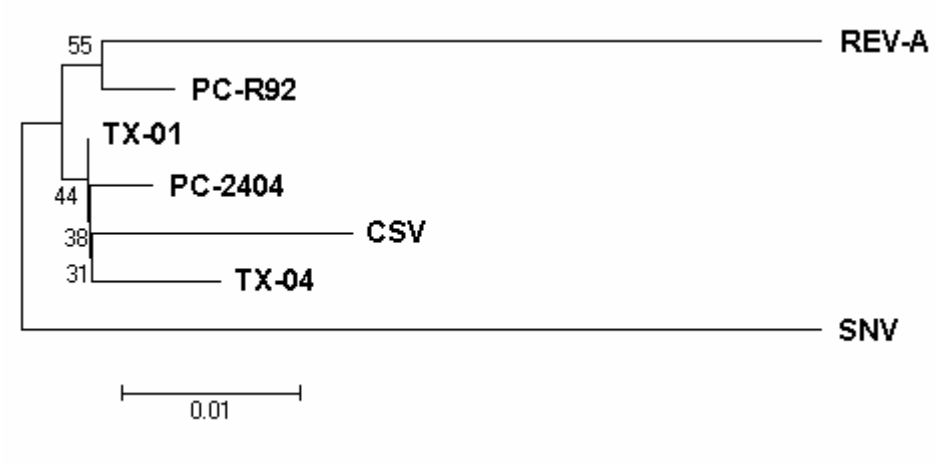


Figure 4. Phylogenetic trees of putative amino acid sequences. The polymerase tree is represented in A and the envelope tree is represented in B. Numbers denote the percentage of 1000 bootstraps in which a certain branch occurred.

DISCUSSION

Whereas loss of habitat due to urbanization is a basic threat to the survival of the prairie chicken, reticuloendotheliosis virus infection, particularly in breeding facilities is a continuing obstacle for rehabitation of the Attwater's prairie chicken. Infected birds typically succumb within a year of infection due to lymphomas or as a result of a secondary infection facilitated by immunosuppression (personal observations). The purpose of this study was to determine the genetic relationship of the prairie chicken virus with other strains of REV. It was surprising that all REV strains, regardless of avian origin or the year isolated, were more than 93% identical. It would seem that the REV genome is stable and well-adapted to a number of avian species. The nucleotide sequences among the CSV group consisting of viruses isolated in 1969 (CSV), 1994 (PC-R92) and early 2000's (TX-01, TX-04 & PC-2404) were 98% or more identical, regardless of the year of isolation. Whereas the duck derived SNV was distinctly more divergent, the REV-A was relatively close to the CSV group. It should be noted that isolates from additional avian species were not sequenced and thus their relationships to these strains are not known. It is possible that differences in habitat and avian host species may influence genetic subtypes of REV strains. The separation of the CSV-REV-A strains with SNV could be attributed to differences in the species, that is galliforms (grouse or prairie chicken and domestic chickens) and anseriforms (ducks).

Although variations in pathogenicity (Purchase and Witter, 1975), antigenicity (Chen et al., 1987; Cui et al., 1986) and *in vivo* replication (Alphandary, 1997) among

various REV isolates have been analyzed, genetic analyses had not been reported. Previous relationships among the various REV isolates have been established through comparison of neutralizing sera and monoclonal antibodies (Chen et al., 1987; Cui et al., 1986). Analysis of several REV isolates revealed three distinct subtypes designated as 1 (REV strain T), 2 (SNV and DIAV) and 3 (CSV), and that subtypes 1 and 2 were the most antigenically different (Chen et al., 1987). Our phylogenetic analyses suggested that only the duck derived SNV was divergent enough to constitute a distinct group. However, the availability of sequences from more REV-A strains might result in the classification of a distinct REV-A group as suggested by antigenic studies. Using nucleic acid and amino acid sequence analyses, this study provides evidence of the close genetic relationship among the various REV strains and that the prairie chicken REV strains have even closer ancestry with the CSV and domestic chicken strains also isolated from Texas.

However, the natural reservoir of the prairie chicken, or for that matter other strains of REV is not known. REV has been associated with poxvirus (Calvert et al., 1993; Hertig et al., 1997) and often with the initial outbreaks of infection in the prairie chicken breeding facilities (personal observations, Drew et al, 1998). The two Texas chicken REV isolates in this study were isolated from CAMS infected with poxvirus derived from pox lesions. In 1998, REV was detected by PCR from a lymphoma-like lesion underlying a pox lesion from a naturally infected domestic turkey submitted to the diagnostic laboratory. (Linares unpublished data, 2005). Furthermore, it has been shown by several investigators that replication competent REV can integrate into poxviruses

and thus provide a convenient vector for transmission (Calvert et al., 1993; Hertig et al., 1997). The poxviruses associated with these infections have not been classified and so nothing is known of their phylogeny nor whether these REV genomes were integrated into the poxvirus genome. However, the role of poxviruses would seem to be critical in the transmission of naturally occurring REV infection.

Because REV and poxviruses have been shown to be vectored by arthropods, insects could provide a convenient mechanism for transmission (Motha, Egerton, and Sweeney, 1984). Transmission of REV has been associated with physical contact (Larose and Sevoian, 1965; Motha, 1984; Paul et al., 1977), such as fecal and bodily fluid contamination (Bagust, 1979; Witter, Smith, and Crittenden, 1981). Mechanical transmission by mosquitoes demonstrated in *Culex annulirostris* could explain the increase in seroconversion of poultry flocks witnessed in summer months (Motha, Egerton, and Sweeney, 1984), as well as the rise in REV positive Attwater's that occur during the same time period (unpublished data). REV also has been isolated from two other species of mosquitoes, *Triatoma infestans* and *Ornithodoros moubata*, following feeding on infected birds (Thompson, Fischer, and Luecke, 1968; Thompson, Fischer, and Luecke, 1971).

REV may use different modes of transmission depending on whether virus is traveling between flocks or among individuals within a flock. An interesting question is what the impact of this versatile means of transmission might have on the seeming genetic stability of these viruses. The natural reservoir(s) of the virus is not known.

However, virus has been isolated from any number of species of birds, any of which could serve as a potential natural reservoir.

CHAPTER III

DEVELOPMENT OF REAGENTS

INTRODUCTION

Reticuloendotheliosis virus was first reported in the endangered Attwater's prairie chicken in 1993 at Texas A&M University (Drew et al., 1998). The resulting disease subsequent to infection has caused depletions in numbers in these birds and thus, potential loss of genetic variability. Previous REV infections have been described in chickens (Hoelzer, Franklin, and Bose, 1979), turkeys (Robinson and Twiehaus, 1974), and ducks (Trager, 1959), as well as several other avian species with the resulting disease severity ranging from relatively low mortality rates in chickens, although many are culled prior to death (Witter et al., 1979), to development of extensive lymphomas (Dren et al., 1988b; Witter, Smith, and Crittenden, 1981). Understanding the disease caused by this virus in the endangered prairie chicken population is critical for designing future methods of prevention.

Preliminary research of REV in the prairie chicken involved development of a set of reagents capable of identifying the virus, and prairie chicken specific antibodies and lymphocytes. Unlike commonly studied animal models, such as the chicken and mouse, commercial reagents developed to recognize prairie chicken antigens do not currently exist. Commercial monoclonal antibodies recognizing chicken CD4 and CD8 antigens were identified that cross reacted with prairie chicken CD4's and CD's (Ferro, 2001).

However, monoclonal antibodies identifying prairie chicken IgY had not been developed. Future studies involving the investigation of prairie chicken antibody responses to REV would require reagents capable of identifying these molecules.

Additionally, few reagents identifying REV were available prior to beginning this study. Chicken anti-REV antisera (Charles River Laboratories, Wilmington, MA) was commercially available, but antibodies specific for REV antigens necessary for establishing standardized assays for the detection of REV were not available. A purified REV protein was also necessary for establishing standardized controls, as well as generating REV antibodies.

In this chapter, the development and purification of reagents that identify REV antigens and prairie chicken antibodies are described. Following the purification of these reagents, enzyme-linked immunosorbent assays (ELISA's) were developed for detection of both virus and prairie chicken antibody.

MATERIALS AND METHODS

Cloning, expression, and purification of gag

Cloning. cDNA was amplified from virus RNA collected from chicken embryo fibroblasts (CEF) infected with REV isolate R92 using the SuperScript First Strand Synthesis System for RT-PCR (Invitrogen, Carlsbad, CA), which utilizes a poly T primer. A 633 base pair (bp) piece at the C terminal region of the gag gene was

amplified by PCR amplification of the cDNA template using the primers REVF868 (CGGAGTACAGGTCACTGACGAGCG) and SQREVGAGRV-1 (GGGCGGGAGAACCCTGAC). Two microliters (µl) of the cDNA were added to a 50 microliter (µl) reaction containing 36.75 µl water, 5 µl 10X Mg free reaction buffer (Promega, Madison, WI), 3 µl 10 millimolar (mM) $MgCl_2$, 1 µl 10 mM dNTP mix, 1 µl of forward primer, 1 µl reverse primer and 0.25 µl Taq polymerase (Promega, Madison, WI). Samples where held at 95º C for 5 min., then cycled at 95º C for 30 sec., 55º C for 30 sec. and 72º C for 2 min. for 40 cycles. Samples were then held at 72º C for 7 min. before storing at 4º C. The PCR product was cloned into the pET Blue-2 expression vector (Novagen, Madison, WI) by blunt end ligation according to the manufacturer's protocol. The ligation reaction was then cloned into NovaBlue chemically competent cells (Novagen, Madison, WI) and plated on LB agar plates with 50 µg/ml ampicillin, 70 µg/ml of X-gal and 1 millimolar (mM) isopropyl thiogalactoside (IPTG). Colonies were collected the following day and inoculated into 3 ml of LB with 50 µg/ml ampicillin, 34 µg/ml of chloramphenicol and 1% glucose and shaken overnight at 37° C. Plasmids were purified with a GenElute plasmid Miniprep kit (Sigma, St. Louis, MO) according to the manufacturer's protocol and successful ligations were confirmed by PCR.

Expression. A plasmid containing the gag insert was used to transform to Tuner cells (DE3; Novagen, Madison, WI) that were grown on LB with 50 µg/ml ampicillin and 34 µg/ml of chloramphenicol. Colonies were collected the following day and inoculated into 20 ml LB with 1% glucose, 50 µg/ml ampicillin and 34 µg/ml chloramphenicol and shaken overnight at 37° C. The overnight culture (20 ml) was used

to inoculate 980 ml LB (1:50 dilution) with 1% glucose, 50 μg/ml ampicillin and 34 μg/ml chloramphenicol. The culture was shaken at 37° C for 3 hours followed by the addition of 1 mM IPTG for induction of protein expression. The culture was then grown for 5 more hours and bacteria were collected by centrifugation.

Protein purification. Expressed gag polypeptide was purified in batch under native conditions using the nickel-nitrilotriacetic acid (Ni-NTA) beads (Qiagen, Valencia, CA) according to manufacturer's protocol. Briefly, pelleted bacteria were resuspended in 5 ml of lysis buffer (50 mM NaH_2PO_4, pH 8.0; 300 mM NaCl; 10 mM imidazole). Five mg of lysozyme were added and the mixture was incubated on ice for 30 min. Cells were sonicated on ice 8 times for 10 sec at 300 W with a 10 sec cooling period between each burst. Cells were centrifuged at 10,000 x g for 30 min at 4°C to pellet debris. One ml of the 50% NiNTA beads (Qiagen, Valencia, CA) were added to the cleared lysate and shaken at 4°C for 1 hour. Beads were centrifuged out of solution at 12,000 x g for 1 min and washed 3 times with 1 ml of washing buffer (50 mM NaH_2PO_4, pH 8.0; 300 mM NaCl; 20 mM imidazole). Beads were removed from the wash by centrifugation at 12,000 x g for 1 min and the supernatant was removed. Purified polypeptide was eluted from the beads by adding 1 ml elution buffer (50 mM NaH_2PO_4, pH 8.0; 300 mM NaCl; 250 mM imidazole) and centrifuging at 12,000 x g for 1 min. Supernatant containing the purified gag polypeptide was collected and stored at -20°C.

Purification of IgY

Attwater's praire chicken IgY was isolated from 3 ml serum using a T-Gel Purification kit (Pierce, Rockford, IL) according to manufacturer's protocol. Briefly, 261 mg potassium sulfate was dissolved in 3 ml of prairie chicken serum and the mixture was centrifuged at 10,000 x g for 20 min. Clarified supernatant was removed and added to a T-Gel adsorbent column (Pierce, Rockford, IL) following equilibration of the column with 12 ml of T-Gel Binding Buffer (Pierce, Rockford, IL). The T-Gel column was washed with 38 ml T-Gel Binding Buffer before eluting the protein with 36 ml T-Gel Elution Buffer (Pierce, Rockford, IL) in 1 ml fractions. Fractions containing the purified IgY were detected using the Bio Rad Protein Assay (Bio Rad, Hercules, CA) and combined.

Rabbit polyclonal antibody production, purification, and biotinylation

Polyclonal antibodies to REV native gag polypeptide and Attwater's prairie chicken IgY were generated in rabbits (Robert Sargeant, Ramona, CA). Rabbits were inoculated monthly with antigen described above in Freund's adjuvant and blood was collected. Rabbit IgG was purified from sera using a HiTrap protein G HP column (Amersham Pharmacia, Uppsala, Sweden) according to manufacturer's protocol. Eluted antibody fractions were combined and concentrated using a 50 KD Vivaspin 6 centrifugal filter (Vivascience, Hanover, Germany). One mg of biotin (Sigma, St. Louis,

MO) was diluted in 75 µl DMSO before adding to 1 ml of purified antibody. The mixture was incubated at room temperature for 30 minutes before removing excess biotin using a PD-10 desalting column (Amersham Pharmacia, Uppsala, Sweden). Fractions containing the biotinylated antibody were combined and the concentration was determined to be 0.32 µg/µl using the Bio Rad protein assay (Bio Rad, Hercules, CA).

SDS-PAGE and western blot analysis

SDS-PAGE. Samples were mixed with 2X Laemmli sample buffer and heated at 95° C for 5 minutes prior to SDS-PAGE. SDS-PAGE was run in a mini-Protean Electrophoretic Apparatus (BioRad Laboratories, Hercules, CA, 94547) on a 12.5% Tris-glycine (acrylamide) gel with a 4% stacking gel in 1X SDS-PAGE running buffer (25 mM Tris; 250 mM glycine, pH 8.3; 0.1% SDS) at 150 volts for 45 minutes. Kaleidoscope high molecular weight protein standards were used for size comparisons (BioRad Laboratories, Hercules, CA, 94547). Gels were stained with 0.25% Coomassie Blue R (Sigma, St. Louis, MO 63178) in 50% methanol and 10% acetic acid. Gels were destained with 50% methanol and 12.5% acetic acid and then stored in water to allow complete rehydration. Duplicate gels were run for staining and western blot analysis.

Western blot. Gels were sandwiched with a piece of nitrocellulose between 2 pieces of Whatman 3mm filter paper that were placed in a western blot cassette and submerged in western transfer buffer (6 g Tris-base/L; 28.8 g glycine/L; 0.1 g SDS/L; and 200 ml methanol/L) in the electrophoresis apparatus. The transfer was conducted at

65 volts for 1 hour. Following transfer, proteins were visualized with Ponceau S (BioRad Laboratories, Hercules, CA) and marked with a pencil for future reference. Lanes were cut into individual strips for exposure to antibody.

Rabbit anti-gag western. The nitrocellulose was blocked overnight in 3% bovine serum albumin (BSA) in tris-buffered saline (TBS). Following blocking the nitrocellulose was washed 4 times in TBS-T (50 mM Tris-HCl pH7.5, 200mM NaCl and 0.02% Tween 20) and one time with TBS (50 mM Tris-HCl pH7.5 and 200mM NaCl). Rabbit anti-gag sera was diluted in TBS and 1 ml of each dilution was incubated with nitrocellulose strips for 1 hour at room temperature. After washing the nitrocellulose 4 times with TBS-T and one time with TBS, secondary antibody goat anti-rabbit conjugated with alkaline phosphatase was diluted in TBS at 1:2500 and 1 ml of the dilution was incubated with each strip of nitrocellulose at room temperature for one hour. The nitrocellulose was again washed 4 times with TBS-T and one time with TBS before developing each strip in 1 ml of 1 step NBT/BCIP (KPL, Gaithersburg, MD) for 10 minutes. The development reaction was stopped with water.

Chicken/prairie chicken anti-REV western. The nitrocellulose was blocked overnight in 5% non fat powdered milk in PBS. Following blocking, the blot was washed four times in PBST (PBS with .02% Tween 20) and one time in PBS. Chicken or prairie chicken anti-REV sera was diluted in 5% milk-PBS and 1 ml of each dilution was incubated with nitrocellulose strips for 1 hour at room temperature. The nitrocellulose was washed 4 times with PBS-T and one time with PBS. Secondary antibody goat anti-chicken alkaline phosphatase was diluted in 5% milk-PBS at 1:500.

Secondary antibody rabbit anti-APC – biotin was diluted 1:1000 in 5% milk-PBS. One ml of the appropriate secondary antibody dilution was incubated with each strip of nitrocellulose at room temperature for one hour. The Western was again washed 4 times with PBS-T and one time with PBS before incubating the strips receiving the rabbit anti-APC – biotin with streptavidin-horse radish peroxidase (strep-HRPO) diluted 1:5000 in PBS for 30 minutes, at room temperature. Strips were washed 4 times with PBS-T and one time with PBS before developing the alkaline phosphatase labeled strips with 1 ml each of 1 step NBT/BCIP (KPL, Gaithersburg, MD) for 10 minutes or the HRPO labeled samples for 10 minutes in 1 ml each of TMB 1-Component Membrane Peroxidase Substrate (KPL, Gaithersburg, MD). Reactions were stopped with water.

Dot blot assay

The dot blot assay was used to confirm the reactivity of the polyclonal rabbit anti-REV gag with the REV gag polypeptide. The assay was performed on nitrocellulose membranes. Gag antigen was serially diluted in 10 fold increments from 30 μg/well in row 1 to 3 x 10^{-4} μg/well in row 6. BSA was added to each well of Row 7 at a concentration of 25 μg/well. Row 8 consisted of gag antigen added at alternating dilutions; 1:10 in columns 1, 3, 5, 7, 9 and 11 and 1: 10^2 in columns 2, 4, 6, 8, 10 and 12. Following addition of antigen, wells were blocked with 5% milk in TBS for 30 minutes. at room temp. Wells were rinsed with water. Serum from two different rabbits was examined. Rabbit #1 was examined in columns 1 through 6 and rabbit #2 was examined

in columns 7-12. Purified antibody was serially diluted 10 fold in 5% milk in TBS and added to columns A-F as follows: A – 1:10; B – $1:10^2$ … F – 1: 10^6. No antibodies were added to Row 8 columns A,B,E and F. Antibodies were incubated at room temperature for 1 hour and then washed with water. The nitrocellulose membrane was then blocked in 5% milk in TBS at room temperature for 30 min. Goat anti rabbit IgG conjugated with alkaline phosphatase was diluted 1:2500 in TBS and incubated on nitrocellulose for 1 hour at room temperature before washing with water. Antibody binding was visualized by adding 1 step NBT/BCIP (KPL, Gaithersburg, MD) until the color changed. The reaction was stopped with water.

ELISA

Anti-gag ELISA. Fifty µl of unlabeled rabbit anti REV gag in PBS, at a concentration of 20 µg/ml, were added to each well of a 96 well plate and incubated at room temperature in a humid container for 2 hours. The unlabeled gag polypeptide was removed and wells were filled completely with 3% BSA in PBS and left at room temperature in a humid container overnight to block wells from nonspecific binding of protein. BSA was removed and the wells were washed twice with PBST and once with PBS. Plates were stored at -20°C. After thawing, 20 ul 0.5% Triton X-100 were added to each well followed by addition of either 100 µl virus in cell culture media or 100 µl of purified REV gag polypeptide (diluted in PBS from a stock concentration of 10 µg/ml). Plates were incubated in a humid container at room temperature for 2 hours and then

washed 4 times with PBST and one time with PBS. One hundred µl biotinylated rabbit anti-REV gag, diluted to 2 µg/ml in PBS with 3% fetal bovine serum (FBS), were added to each well. Plates were incubated for 30 minutes at room temperature and washed 4 times with PBST and once with PBS before adding 100 µl strep-HRPO, diluted 1:5000 in PBS - 3% FBS, to each well. Plates were incubated for 30 minutes at room temperature and washed 4 times with PBST and once with PBS. One hundred µl of TMB Microwell ELISA substrate (KPL, Gaithersburg, MD) were added to each well and allowed to develop for 10 minutes. Absorbance was read at 630 nm.

Anti-prairie chicken IgY ELISA. Purified REV gag polypeptide was diluted at varying concentrations in either PBS or bicarbonate/carbonate coating buffer (2.93 g $NaHCO_3$, 1.59 g Na_2CO_3, 0.203 g $MgCl_2$ in l liter of water, pH 9.6). Fifty µl of the diluted antigen was added to each well of a 96 well plate and incubated at room temperature in a humid container for 2 hours before removing the protein. Wells were filled completely with 3% BSA in PBS and left at room temperature in a humid container overnight to block against nonspecific binding of protein. BSA was removed and the wells were washed twice with PBST and once with PBS. Plates were stored at -20°C. Prior to adding serum, plates were thawed at room temperature. Prairie chicken serum was diluted to varying concentrations in PBS and 100 µl of the dilution was added to each well. Plates were incubated in a humid container at room temperature for 1 hour and then washed 4 times with PBST and one time with PBS. Biotinylated rabbit anti-prairie chicken IgY was diluted to varying concentrations in PBS – 3% FBS and 100 µl was added to each well. Plates were incubated for 30 minutes at room temperature and

washed 4 times with PBST and once with PBS. Strep-HRPO was diluted 1:5000 in PBS - 3% FBS and 100 µl were added to each well. Plates were incubated for 30 minutes at room temperature and washed 4 times with PBST and once with PBS. One hundred µl of TMB Microwell ELISA substrate (KPL, Gaithersburg, MD) were added to each well and allowed to develop for 10 minutes. Absorbance was read at 630 nm.

RESULTS

Purification of REV gag protein and generation of anti-REV gag polyclonal antibody

REV gag polypeptide was expressed in bacteria as a fusion protein with a carboxyl 6X histidine tag and purified by nickel affinity chromatography under native conditions. Purified gag polypeptide was visualized as a band of approximately 25 kD on an SDS-PAGE gel (Fig. 5). Following purification, polyclonal antibodies specific for the gag polypeptide were produced in rabbits. Western blot analysis indicated the rabbit generated antibody was specific for the gag polypeptide whereas normal rabbit sera, serving as a negative control, did not react with the polypeptide (Fig. 6).

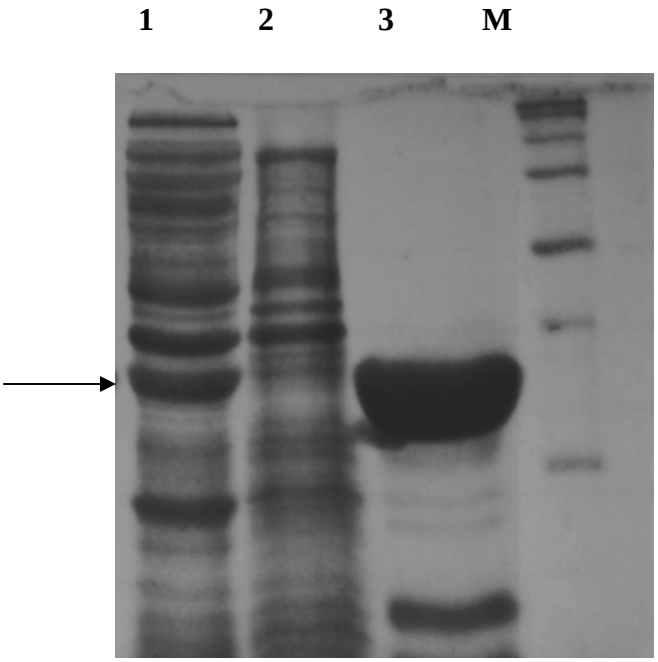


Figure 5. SDS PAGE gel with the purified recombinant gag polypeptide. Lane 1 represents induced bacteria expressing the gag polypeptide as indicated by the arrow Lane 2 represents uninduced bacteria not expressing the gag polypeptide. Lane 3. represents purified gag polypeptide.

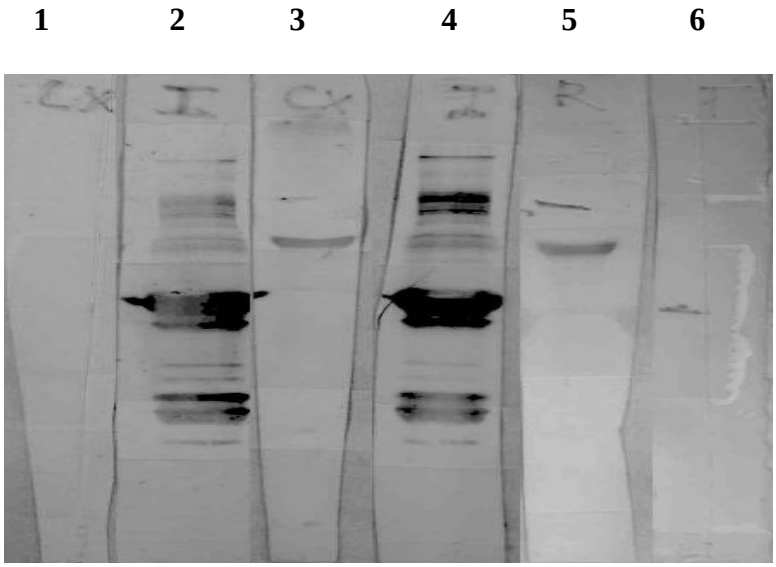


Figure 6. Western blot of purified gag polypeptide with polyclonal rabbit anti-gag antibody. Lanes 1, 3 and 5 represent normal rabbit serum which served as a negative control. Lane 2, 4 and 6 represent purified gag protein. Lanes 1 and 2 were blotted with rabbit anti-gag serum diluted 1:1000. Lanes 3 and 4 were blotted with rabbit anti-gag diluted 1:10000. Lanes 5 and 6 were only blotted with the secondary antibody to serve as controls.

Dot blot analysis

A dot blot assay was used to better determine the specificity of the generated polyclonal antibody for native gag polypeptide (Fig. 7). Sera from two different rabbits were characterized. A dose dependent interaction was observed between the specific antisera and the gag protein. Both antibodies showed specificity for the gag polypeptide as neither of the antibodies reacted with 25 μg of BSA in Row 7. Antibody #2 had a 10 fold higher concentration than that of antibody #1. Rabbit Ab#1 showed reactivity at a 1:100 dilution with 3×10^{-3} μg gag polypeptide and at a 1:1000 dilution with 3×10^{-2} μg gag polypeptide. Rabbit Ab#2 appeared to have a higher concentration as it reacted at a 1:100 dilution with 3×10^{-3} μg gag polypeptide and at a 1:1000 dilution with 3×10^{-4} μg gag polypeptide.

REV antigen ELISA

An ELISA was developed to detect and quantitate virus in REV infected samples. Unlabeled rabbit anti-REV gag antibody was immobilized on a standard ELISA plate. Virus samples were loaded into wells with 0.5% Triton X-100 such that the gag protein could bind to the immobilized antibody. Two fold serial dilutions from 1:16 to 1:512 of both purified REV gag polypeptide in PBS and REV prairie chicken isolate R92 in culture media were analyzed and absorbance at 630 nm was compared (Fig. 8). Uninfected culture media, also serially diluted and used as a negative control, was

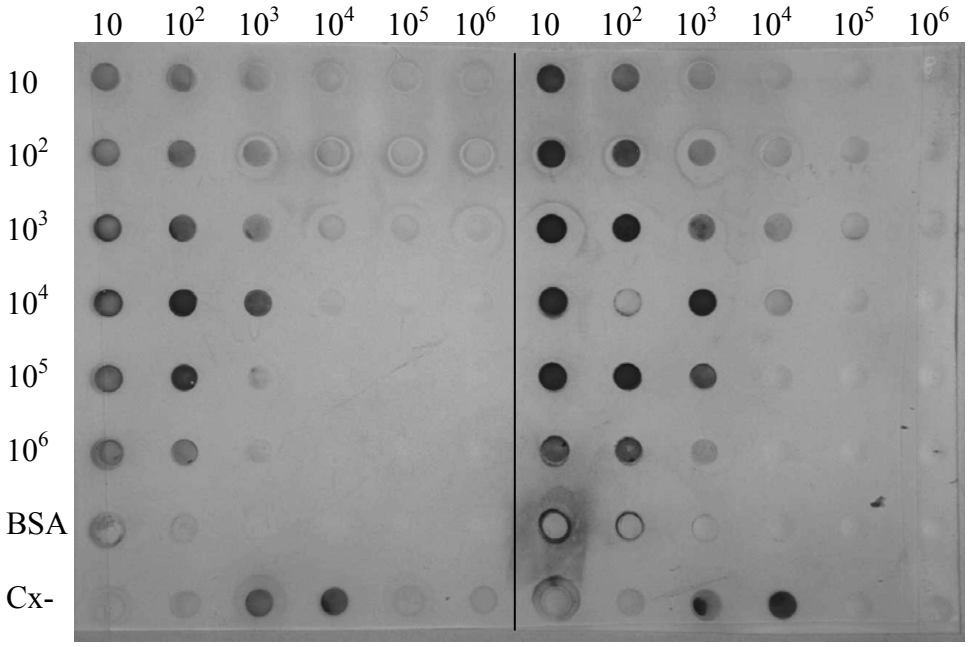


Figure 7. Dot blot confirming reactivity of polyclonal REV gag antibody with gag antigen. Antibody dilutions are shown across the top. Antigen dilutions are shown on the left side in rows 1-6. Row 7 contains BSA at a concentration of 500 ng/µl. Row 8 contains gag antigen – columns A, C and E with a dilution of 1:10 and columns B, D and F with a dilution of 1:100. No primary antibody was added to Row 8 columns A, B, E and F.

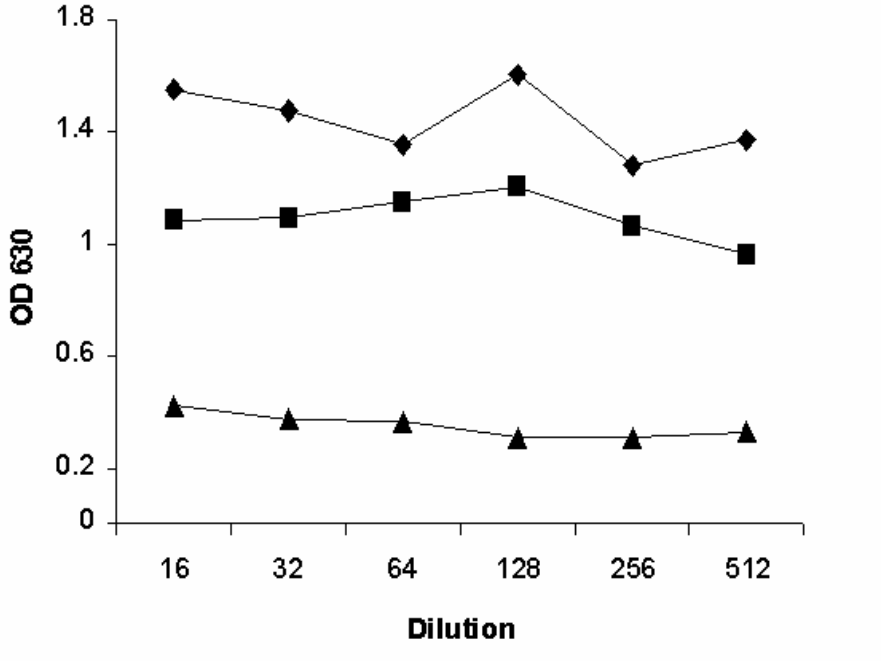


Figure 8. REV antigen ELISA. OD values are compared for varying dilutions of purified REV gag (◆) in PBS, REV isolate R92 (■) in tissue culture media and a blank control (▲). X axis values represent dilution factors from stock concentration. Stock concentration of REV gag was 10 µg/ml. All wells were coated with polyclonal rabbit anti-REV gag prior to addition of the antigen.

treated in the same manner as the infected samples. Purified REV gag polypeptide and viral isolate PC-R92 were both readily detected by the ELISA and show absorbance values over 1.0 through dilutions out to 1:256 as compared with substantially lower absorbance values of the uninfected negative control.

Western blot analysis of rabbit polyclonal antibody against prairie chicken IgY

Prairie chicken IgY was purified from serum and injected into rabbits for production of polyclonal antibody. Purified REV gag polypeptide was transferred to nitrocellulose and reacted with either chicken or prairie chicken serum. Both rabbit anti-prairie chicken IgY and goat anti-chicken Ig were used to detect the presence of antibodies against the IgY to determine cross reactivity (Fig. 9). Prairie chicken anti-REV serum positively labeled the gag antigen and was detected by the rabbit anti-prairie chicken IgY. No cross reactivity was observed between the prairie chicken IgY and the goat anti-chicken Ig. Chicken anti-REV serum also positively labeled the gag antigen and was detected by the goat anti-chicken Ig. Additionally, cross reactivity was observed between the chicken IgY and the rabbit anti-prairie chicken IgY secondary antibody. Secondary antibody alone did not react with the gag polypeptide.

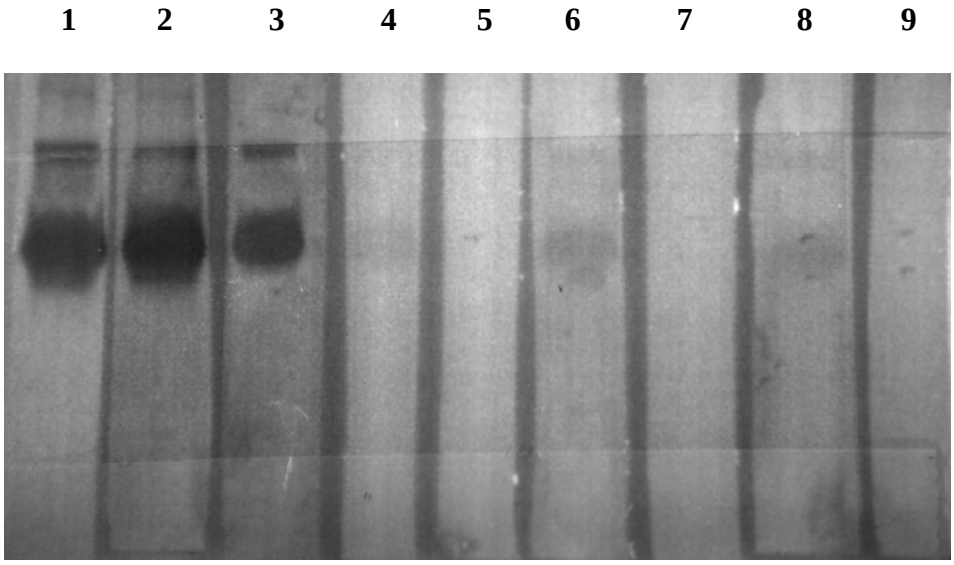


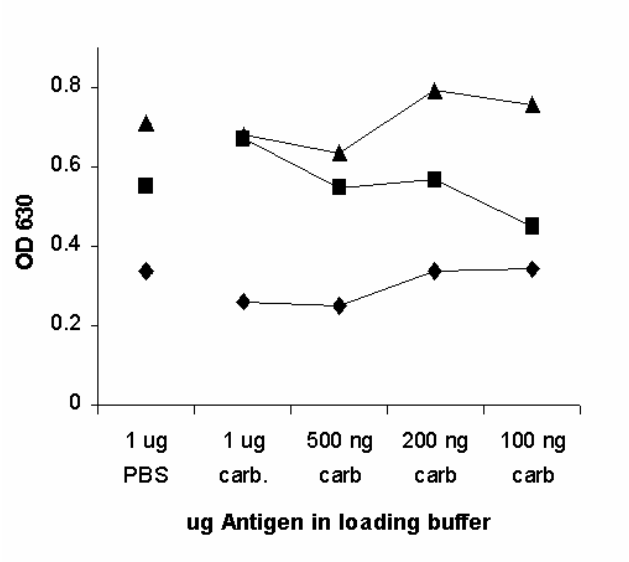
Figure 9. Western blot showing reactivity of REV gag antigen with chicken and prairie chicken antisera. Antigen: REV purified gag antigen was loaded in all lanes. Primary antibody: Lanes 1, 2, 5 and 6 were reacted with chicken anti-REV serum. Lanes 3, 4, 7 and 8 were reacted with Attwater's prairie chicken anti-REV serum. Lane 9 was not reacted with primary antibody. Secondary antibody: Lanes 1 and 4 were incubated with goat anti-chicken alkaline phosphatase antibody. Lanes 2 and 3 were incubated with rabbit anti-APC-biotin. Lanes 5-9 were not labeled with a secondary antibody. 3rd label: Lanes 2, 3, 6 and 8 were labeled with strep-HRPO. All other lanes received PBS. Develop: Lanes 1, 4, 5 and 7 were developed with phosphatase substrate. Lanes 2, 3, 6, 8 and 9 were developed with peroxidase substrate.

ELISA

Coating buffer and antigen concentration

An ELISA to detect the presence of prairie chicken antibodies to REV was developed using REV gag polypeptide immobilized on a 96 well plate. In order to determine the effectiveness of a bicarbonate/carbonate coating buffer versus that of PBS, gag polypeptide was added at concentrations of 1 µg/well, 500 ng/well, 200 ng/well and 100 ng/well in the bicarbonate/carbonate buffer. One µg/well of gag antigen was also added in PBS. Absorbance at 630 nm was compared among all concentrations (Fig. 10). Serum from 3 birds was analyzed - #235 at 10 weeks post infection (pi) with REV, #232 at 19 weeks pi, and #331 was uninfected. Serum was diluted 1:50 (A) and 1:100 (B) in PBS based on preliminary assays. Secondary antibody (rabbit anti-prairie chicken IgY-biotin) concentrations remained constant at 4µg/ml. Serum from the negative bird, #331, showed a lower absorbance with 1 µg of antigen in the bicarbonate/carbonate buffer than in the PBS at both dilutions of primary antibody. Absorbances of the negative bird were lowest at both 1 µg and 500 ng of antigen in the bicarbonate/carbonate buffer. Bird #235 had consistently higher absorbance readings than those of bird #232. Bird #232 reached its highest absorbance values with 1 µg gag in the bicarbonate/carbonate buffer at both primary antibody dilutions. These peak absorbance values coincided with one of the lowest absorbance values of the negative

A.



B.

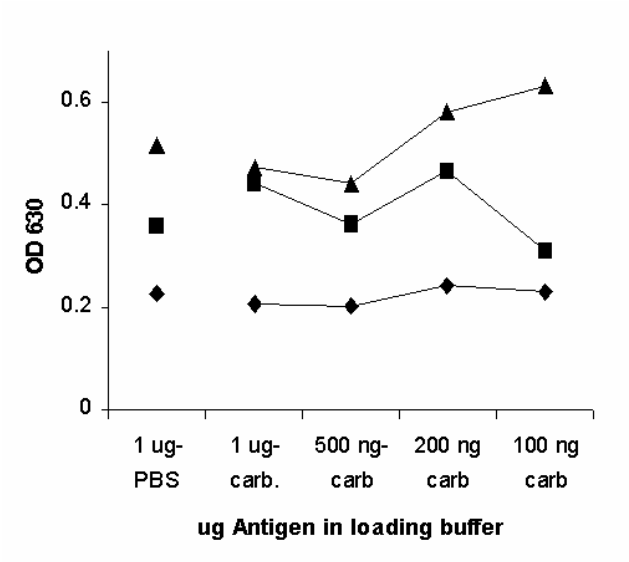


Figure 10. REV antibody ELISA. OD values are compared for varying concentrations of purified REV gag coated on the plate. Sera from two REV positive birds, #235 (◆) and #232 (■) were compared as well as one REV negative bird, #331 (▲), which served as a negative control. X axis values represent total amount of gag diluted in the specified buffer – either PBS or bicarbonate/carbonate buffer. Primary antisera was diluted 1 to 50 (**A)** and 1 to 100 (**B)** in PBS.

control. Thus, 1 μg antigen in the bicarbonate/carbonate buffer was identified as the ideal antigen coating condition for this assay.

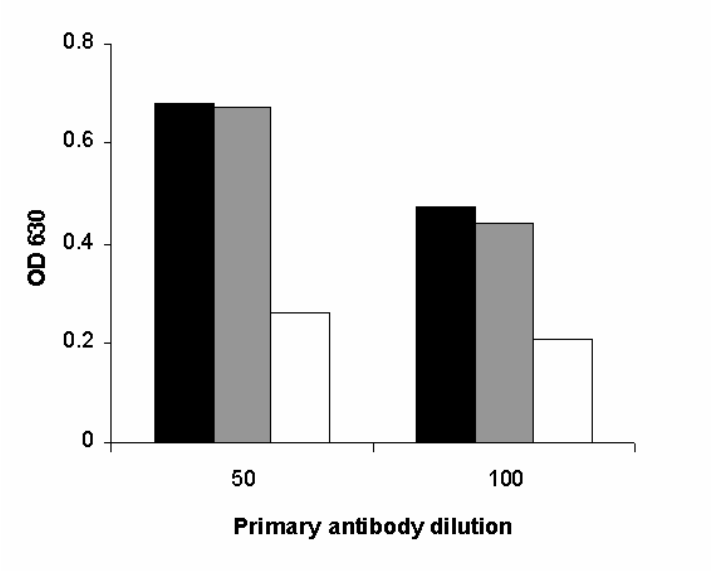
Primary antibody concentration

Absorbance values were compared for primary antibody dilutions of 1:50 and 1:100 and a negative control using 1 μg antigen coated on plates in the bicarbonate/carbonate buffer (Fig. 11). Sera from both positive birds resulted in higher absorbance values at a 1:50 dilution than with a 1:100 dilution (A). Compared with their respective controls, sera from both positive birds resulted in a greater difference at a 1:50 than at 1:100 (B), thus identifying the preferred primary antibody dilution as 1:50.

DISCUSSION

An ELISA identifying REV envelope proteins has been previously developed for domestic chickens (Cui et al., 1988). However, as the antigenicity of the prairie chicken REV isolate has not been compared with other RE viruses, cross reactivity of the previously described assay with the prairie chicken REV isolate was unknown. Additionally, reagents used in the development of the previously described assay were unavailable. This chapter describes the development of a unique assay for detection of the prairie chicken REV isolate. Due to potential variability in the envelope region, this assay was targeted at detection of the more conserved nucleocapsid and capsid proteins

A.



B.

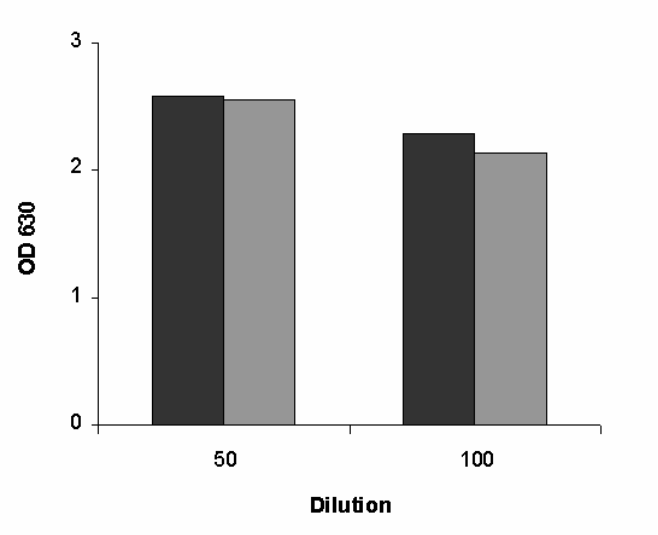


Figure 11. Comparison of REV antibody ELISA absorbance values. Absorbance is compared at varying primary antibody dilutions (A) for bird #235 (■), #232 (▒) and the negative control, #331(□). Fold difference of absorbance values were compared for birds #235 (■) and #232 (▒) with the negative control (B).

of the gag gene. By using a more conserved region of the virus, this assay has a greater potential for cross reactivity with other REV strains. Previous RNA virus ELISA's have been developed against nucleocapsid proteins in vesicular stomatitis virus, rabies, mumps, Newcastle disease virus, and infectious bronchitis virus (Ahmad, 1993; Errington, 1995; Hasselblom, Linde, and Ridell, 2004; Hummel, 1992; Linde, 1987; Reid-Sanden, 1990).

Original plans for development of the REV gag specific ELISA were aimed at using the whole gag encoded protein However, attempts at expression of the entire gag gene in a bacterial expression system were unsuccessful. Subsequent attempts were made to express the N terminal 40%, a middle region encompassing 60% of the gag gene and the C terminal 40%. Attempts to express the N terminal region and the middle region were unsuccessful, however, expression of the C terminal region was achieved. The inability to express these other regions may be the result of a toxic property of the N terminal region of this protein, preventing its expression in bacteria. As expression of the C terminal region of the gag gene was successful, this assay was developed to identify a bacterial expressed polypeptide encoded by this region that included the whole nucleocapsid and a partial capsid protein.

Development of a practical ELISA for detection of REV infection required the ready availability of a stock of REV antigen necessary for standardization of the assay and for positive controls. An added benefit of an easily reproducible system of expression and purification of the antigen could be inexpensive, large scale production of ELISA reagents.

Polyclonal antibodies shown to react with the recombinant purified gag polypeptide used for antibody production, also reacted with antigen on virus grown in productively infected cell culture. The development of the ELISA using this antibody and antigen combination provides for extensive screening of REV in prairie chicken populations. In addition, titration of lab stocks of the prairie chicken isolates was readily achieved by screening $TCID_{50}$ media sample dilutions as positive or negative for virus.

This assay has only been analyzed with the prairie chicken isolate of REV. Determination of the cross-reactivity of the rabbit polyclonal antibody with other REV subtypes has not been made. However, cross-reactivity among REV subtypes with the envelope ELISA developed by Cui et al. (1988) lends strong support for the possibility that this gag antigen ELISA will cross-react with gag polypeptides of other subtypes.

REV infectivity studies in chickens and turkeys have revealed the antibody responses in these birds following infection (Bulow, 1977; Ianconescu, 1978; Larose and Sevoian, 1965; McDougall, 1980; Witter, Smith, and Crittenden, 1981). In order to identify antibody responses of prairie chickens to REV, a method for prairie chicken IgY detection was required. Cross reactivity of antibodies recognizing chicken immunoglobulin with prairie chicken IgY was not observed, thus necessitating the development of reagents to identify the prairie chicken IgY. Polyclonal antibody developed in rabbits successfully labeled prairie chicken antisera bound to purified REV gag polypeptide. Interestingly, the antibody was also capable of cross-reacting with chicken antisera although no cross-reactivity was seen with anti-chicken immunoglobulin and the prairie chicken IgY common antigen.

Development of an ELISA to detect prairie chicken antibodies to REV was based on immobilization of the purified REV gag antigen. Comparison of two coating buffers indicated that higher absorbance values in positive birds coinciding with the lowest background levels from antibody negative birds was achieved using the bicarbonate buffer rather than PBS. Primary antibody dilutions at 1:50 achieved the greatest best ratio when comparing positives with negatives. Although not shown in the data, secondary rabbit anti-prairie chicken IgY labeled with biotin was most effective at a dilution of 3-4 μg/ml. The development of reagents specific for prairie chicken immunoglobulin, as well as the prairie chicken REV isolate, provided reagents for the studies in this dissertation and will be useful for future investigations into immune responses, pathogenesis, REV host cell specificity and ultimately, vaccine trials.

CHAPTER IV

THE USE OF FLOW CYTOMETRY TO DISCRIMINATE AVIAN LYMPHOCYTES FROM CONTAMINATING THROMBOCYTES*

Evaluation of peripheral blood mononuclear cells (PBMC) in avian species by flow cytometry is complicated by the presence of large numbers of nucleated thrombocytes. With light scattering properties similar to those of lymphocytes, variations in the proportion of thrombocytes in PBMC can result in apparent shifts in percentages of lymphocyte subpopulations. We have applied a dual-labeling procedure for flow cytometric analyses to exclude thrombocytes from the analyzed population by labeling with K55 monoclonal antibody (MAb), which differentially labeled leukocyte and thrombocyte populations. Flow cytometric analyses with K55 labeled chicken PBMC differentiated leukocytes into three populations according to their intensity of fluorescence. Using the K1 MAb, the K55-low population was shown to consist of thrombocytes. Dual-labeling with K55 MAb and MAb specific for B lymphocyte, CD4 or CD8 markers indicated that the K55 intermediate population consisted of lymphocytes. Therefore, concentrations of $CD4^+$ and $CD8^+$ T lymphocytes could be determined from the lymphocyte fraction by gating specifically on the K55 intermediate cells. Selecting cross-reactive chicken MAbs that included K55, this protocol was

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shown to identify $CD4^+$ and $CD8^+$ T lymphocytes in PBMC of another avian species, the endangered Attwater's prairie chicken.

INTRODUCTION

Flow cytometry is a preferred method to phenotype *ex vivo* derived individual leukocytes. Changes in concentrations of individual lymphocytes are indicative of pathogen-host interactions. While B lymphocytes generate pathogen-specific antibodies, T lymphocytes with the CD4 marker are critical for regulating lymphocyte functions and those with the CD8 marker are critical for controlling acute viral infection (Pei, Briles, and Collisson, 2003; Seo and Collisson, 1997; Seo et al., 2000; Seo et al., 1997). Monoclonal antibodies (MAbs) available for a number of chicken leukocyte markers, including mammalian homologues for the lymphocyte CD4, CD8, CD3 and Bu-1 molecules (Chan et al., 1988; Chen et al., 1986; Veromaa et al., 1988) have been used to identify cells expressing these antigens by flow cytometry. B lymphocytes can be similarly monitored by the presence of cell surface Bu-1 molecule (Veromaa et al., 1988).

A difficulty in quantification of avian leukocyte populations is the presence of abundant nucleated thrombocytes in peripheral blood mononuclear cells (PBMC). The differentiation of thrombocytes from lymphocytes, in particular, would facilitate determination of absolute and relative numbers of lymphocyte types and subtypes. Nicholson et al. (Nicholson, Jones, and Hubbard, 1993) described a method of three

color staining for analysis of CD4 populations in AIDS patients by adding CD45, a common leukocyte marker, to samples to allow gating with improved purity of the lymphocyte population.

An avian MAb K55 labels a chicken pan-leukocyte marker, differentiating lymphocytes from thrombocytes (Chung, Lillehoj, and Jenkins, 1991). This study shows that dual-labeling with K55 and specific lymphocyte markers could be used to identify B, T cells and T cell subtypes in flow cytometric analyses. The versatility of this procedure was also demonstrated in its application using cross-reactive chicken MAbs to distinguish these populations of cells from an endangered species of grouse, the Attwater's prairie chicken.

MATERIALS AND METHODS

Birds and blood collections

Leghorn chickens used for these studies were housed at the Texas A&M University Poultry Science Center. Attwater's prairie chickens and the greater/Attwater's F1 crosses were hatched and maintained at the Small Upland-bird Research Facility (SURF) located at Texas A&M University, College Station, Texas. Two to 3 ml of EDTA-anticoagulated blood was collected by jugular venipuncture, as approved by the University Laboratory Animal Care Committee, Texas A&M University, College Station, Texas.

Separation of peripheral blood mononuclear cells (PBMC)

Blood was diluted 1 to 1 in Alsever's solution and PBMC were separated using Histopaque-1077® according to the manufacturer's instructions (Sigma Diagnostics, St. Louis, MO), with the exception of adding bovine serum albumen (BSA) to the wash buffer (Li et al., 2000). Briefly, at room temperature, 5 ml aliquots of Histopaque®-1077 were overlaid with the blood/Alsever's mixture and centrifuged for 30 minutes at 400 x g. Following centrifugation, the opaque interface was collected and washed twice at 4° C with phosphate buffered saline containing 0.2% bovine serum albumin and 0.2% sodium azide (PBA) before centrifuging for 10 minutes at 250 x g and 4°C (Li et al., 2000). Cell numbers were calculated and the cell concentrations adjusted to a 1 x 10^6 cells/ml for indirect fluorescent antibody labeling.

Monoclonal antibodies (MAb)

Purified CT4 MAb, specific for the CD4 marker, and CT8 MAb and 3-298 MAb, specific for the CD8 marker, were obtained conjugated with FITC from Southern Biotechnology Associates, Inc. (Birmingham, AL). A panel of chicken-specific MAbs was evaluated to select for cross-reactivity for prairie chicken PBMC. Cell culture supernatants of CD8 specific MAbs 11-39, 11-38, 11-13, 11-9, 9-8, EP72, 3-110, 3-157, 3-292, and 3-298; and cell culture supernatants of CD4 specific MAbs 2-6, 2-35, 7-125, and 10-3 were generous gifts from Drs. Luhtala and Vainio, University of Turku,

Finland (Luhtala, 1998; Luhtala et al., 1995; Luhtala et al., 1997; Luhtala et al., 1993; Paramithiotis, Tkalec, and Ratcliffe, 1991). The K1 MAb, a reported macrophage and thrombocyte marker (Kaspers, Lillehoj, and Lillehoj, 1993) and K55 MAb, a reported leukocyte common marker (Chung, Lillehoj, and Jenkins, 1991) were generous gifts from Dr. Hyun Lillehoj (USDA-ARS, Beltsville, MD). Mouse IgG2b (Southern Biotechnology Associates, Inc.) was used as an irrelevant antibody control.

Biotinylation of K55

The K55 MAb was purified from mouse ascites, using a HiTrap protein G HP column (Amersham Pharmacia, Uppsala, Sweden). Eluted antibody fractions were combined and concentrated using a 50 KD Vivaspin 6 centrifugal filter (Vivascience). One milligram of biotin (Sigma, St. Louis, MO) was diluted in 75 µl DMSO before adding to 1 ml of purified antibody (modified from Shevach, 1992)(Shevach, 1992). The mixture was incubated at room temperature for 30 minutes before removing excess biotin using a PD-10 desalting column (Amersham Pharmacia, Uppsala, Sweden). Fractions containing the biotinylated antibody were combined and the concentration was determined to be 0.32 µg/µl using the Bio Rad protein assay (Bio Rad, Hercules, CA).

Flow cytometry

PBMC were separated by density gradient centrifugation in Ficoll-hypaque (Histopaque; Sigma, St. Louis, MO) (Seo and Collisson, 1997). All cell incubations and washes were done at 4°C. Prior to labeling with specific MAb, nonspecific binding by cellular Fc receptors was blocked by incubating 10^6 PBMC for 10 min at 4^o C with a final concentration of 2mg/ml of normal goat IgG (Sigma Diagnostics, St. Louis, MO) (Li et al., 2000). Aliquots of 50 µl with 10^6 cells, suspended in PBA, were mixed with 50 µl of MAb hybridoma supernatant or 50 µl of a 1:25 dilution of purified MAb for 30 min at 4^o C. The cells were washed twice with PBA, before incubating for 30 min with 50 µl of a 1:25 dilution of goat anti-mouse fluorescein isothiocyanate (FITC) labeled secondary antibody (Kirkegaard & Perry Laboratories, Inc., Gaithersberg, MD). K55 MAb and CD4 or CD8 specific MAbs used for dual labeling of cells were directly labeled. Fifty µl of biotinylated K55 (diluted 1:50) and CD4 or CD8 specific MAb (diluted 1 part in 50 parts buffer) were added simultaneously to 50µl incubated for 30 minutes on ice. After washing the cells with PBA, streptavidin conjugated R-phycoerythrin Cy5 (0.2 mg/ml stock) diluted 1 part to 75 parts buffer was incubated with the cells in order to detect bound biotinylated K55 MAb. After 30 minutes incubation, the cells were washed thoroughly with PBA, resuspended in 200 µl 1% paraformaldehyde and incubated for 30 minutes at 4^o C. Following paraformaldehyde fixation, cells were washed with 4 ml of PBA, resuspended in 200 µl of PBA, and stored at 4^oC. Controls were included for secondary antibody and normal primary sera. Flow

cytometric analyses were performed within 24 to 48 hours of sample processing with a FACSCalibur™ (Becton Dickinson, San Jose, CA) by the Core Flow Cytometry Facility at Texas A&M University. Florescence compensation was performed in hardware; however, spectral overlap was minimal for the combination of fluorescein and PE-Cy5. A minimum of 3,000 events were collected for each sample. Data were analyzed using FlowJo™ (TreeStar, Inc., Ashland, OR).

RESULTS

The K55 leukocyte specific MAb differentially labels three populations of PBMC

Flow cytometric analyses have indicated that the $CD4^+$ and $CD8^+$ single-labeled cell populations make up as low as 4 and 2%, respectively, of the leukocyte population in chicken PBMC, and using light scatter gating, an overwhelming number of nucleated cells are thrombocyte-like (data not shown). Because the K55 MAb had been reported as a pan-leukocyte marker (Chung, Lillehoj, and Jenkins, 1991), we used this antibody to establish a gating strategy to eliminate thrombocyte contamination of the leukocyte-gated cells, similar to that used to determine human lymphocyte subtypes (Nicholson et al., 1996; Nicholson, Jones, and Hubbard, 1993). Figure 12 shows that K55 labeling of chicken leukocyte preparations identified three populations differentiated by their

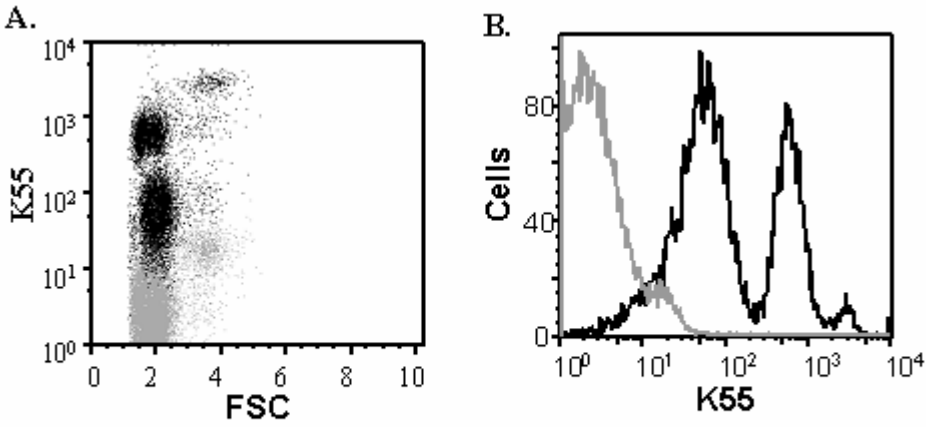


Figure 12. K55 labeling of chicken PBMC using flow cytometry. A. dot plot shows K55 fluorescence graphed against forward scatter of cells. Populations in black represent K55 labeled cells as compared with an unlabeled control cells shown in gray. Values on the x axis represent PMT voltages times 100. B. Histogram of the samples as shown in A with K55 label represented in black and the unlabeled control cells shown in gray.

intensity of staining (populations in black), as compared with a sample of cells unlabeled with K55 (population in gray).

Thrombocytes express low levels of K55

Because the K55-low population was the largest in cell numbers and would best correspond with the absence of K55 expression by thrombocytes reported by Chung et al (Chung, Lillehoj, and Jenkins, 1991), it was deduced that the thrombocytes expressed this low level of fluorescence. Verification that the K55-low population was thrombocytes was shown by dual-labeling of cells with K1, a macrophage and thrombocyte marker (Kaspers, Lillehoj, and Lillehoj, 1993) and the K55 MAb (Fig. 13B). All $K1^+$ cells, shown in Figure 13B, expressed only a low level of K55. Based on the presence of K1 on the K55-low cells, this population was considered to consist of thrombocytes, corresponding to the population expressing the K1 marker, but not expressing detectable levels of K55 as described by Chung et al. (Chung, Lillehoj, and Jenkins, 1991). The cells with intermediate expression of K55 did not express the K1 marker (Fig. 13B). Consistent with the reported absence of K1 on lymphocytes, leukocytes with intermediate expression of K55 were likely to be lymphocytes. A small population of K55-low cells exhibiting a higher forward scatter than the thrombocytes can be seen in Figure 13B (middle column). This side population is present to some extent in each of the samples and is assumed to represent thrombocyte aggregates. By

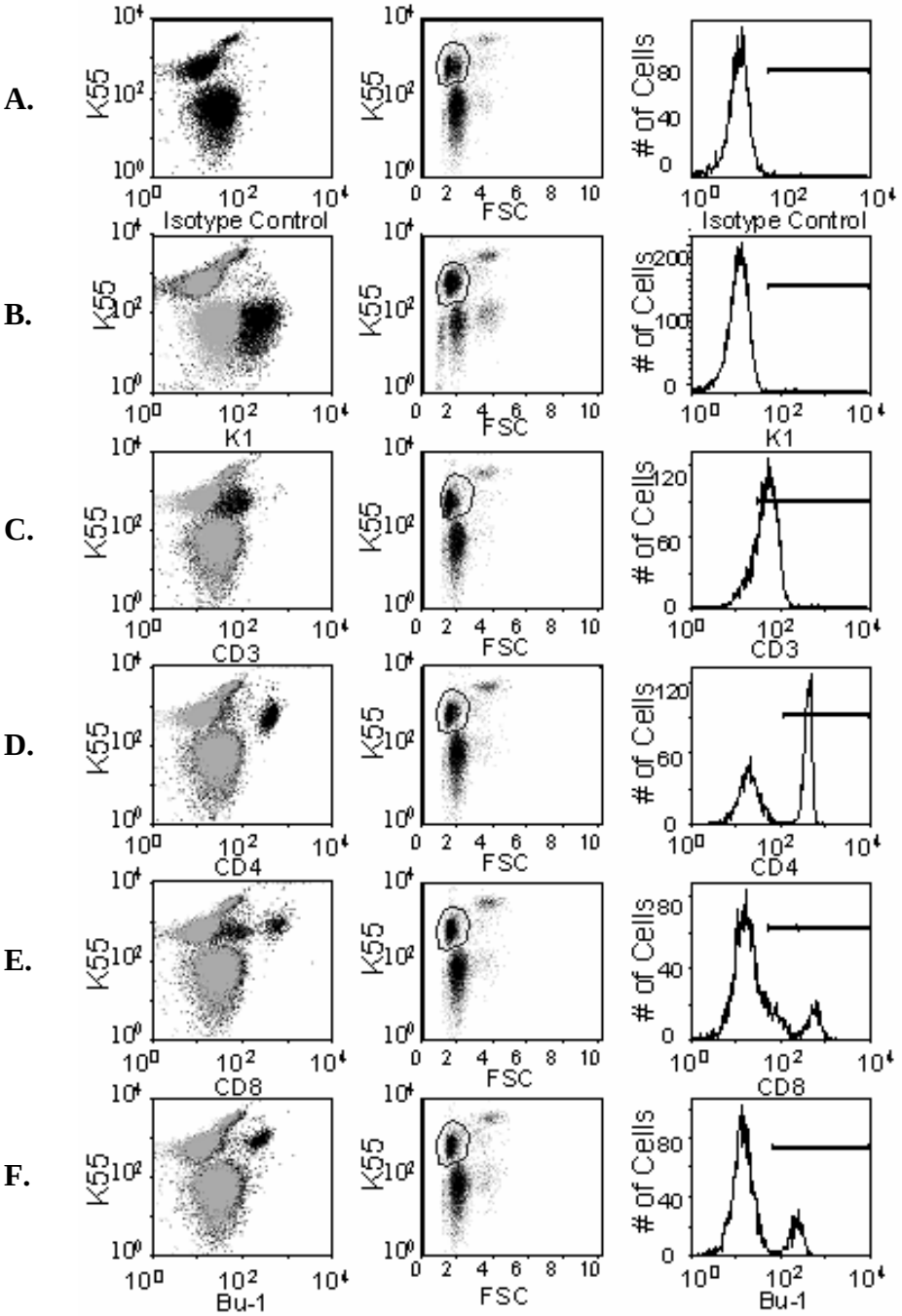


Figure 13. Flow cytometry of PBMC dual labeled with K55 and various leukocyte markers. Column on left shows dot plots of K55 fluorescence (y axis) versus fluorescence of various markers (x axis). The middle column shows dot plots of K55 fluorescence (y axis) graphed against forward scatter (x axis). Values on the x axis in the middle column represent PMT voltages times 100. The polygons were regions used to gate on the lymphocyte populations shown in the histograms in the column on the right. Rows are designated as follows: K55 vs. A) Irrelevant antibody control, B) K1, C) CD3, D) CD4, E) CD8, F) Bu-1. B-F are overlays with cells stained only with K55.

gating on that population, we find that they were only labeled with the K1 antibody (data not shown).

All Bu-1$^+$, CD4$^+$ and CD8$^+$ cells express intermediate levels of K55

In order to demonstrate that the K55 intermediate cells were indeed lymphocytes, cells were dual-labeled with K55 and MAbs specific for either the T or B lymphocyte markers, CD3 or Bu-1, respectively. T cell populations were further determined using MAbs for the CD4 and CD8 lymphocyte markers. All Bu-1$^+$, CD4$^+$ and CD8$^+$ cells expressed intermediate levels of K55 (Fig. 13D-F). The relative percentages of cells expressing Bu-1, CD4 or CD8 were then determined as a fraction of the K55-intermediate cells in the region containing lymphocytes. Because the absolute numbers and ratios of CD4$^+$ and CD8$^+$ T lymphocytes are often an indication of pathogen-host immune interaction, the relative ratios of CD4$^+$ and CD8$^+$ lymphocytes from chickens were also calculated (Table 5). Variations in the lymphocyte subpopulations are shown among four chickens analyzed. CD4$^+$ percentages ranged from 62.1-71.8%, whereas CD8$^+$ percentages ranged from 16.6-33.1%. Calculation of CD4$^+$/CD8$^+$ ratios resulted in a range from 2.17-3.91.

The K55-high population did not co-express the K1 marker nor did it express any of the lymphocyte markers, and therefore, were not considered either thrombocytes or

Table 5. Percentage of labeled cells in each defined population compared with the total number of cells labeled.

	Chicken Identifier[1]			
Labeled MAbs[2]	A	B	C	D
K55 Low[3]	60.6	37.5	45.8	55.9
K55 Intermediate	24.2	50.7	46.0	34.0
K55 High	11.3	6.8	4.8	5.6
CD4/K55 Intermediate	62.1	63.3	64.1	71.8
CD8/K55 Intermediate	17.6	25.1	16.6	33.1
CD3/K55 Intermediate	81.9	73.2	75.0	77.0
Bu-1/K55 Intermediate	10.3	17.2	16.7	13.5
K1/K55 Intermediate	3.04	2.13	3.97	2.9
CD4/CD8 Ratio[4]	3.53	2.52	3.91	2.17

[1] Individual chickens used for preparation of peripheral blood mononuclear cells

[2] PBMC were labeled with mouse K55 MAb conjugated to PE and CD4 or CD8 antigen specific
MAbs labeled with FITC

[3] Refers to intensity of fluorescence from K55 MAb labeling

[4] Ratios of cells dual labeled with K55 and CD4 MAbs to cell labeled with K55 and CD8 MAbs

lymphocytes. As indicated by the high level of autofluorescence, these leukocytes with high levels of K55 expression were thought to include monocytes and/or granulocytes.

Chicken MAb specific for cells expressing CD8 and CD4 markers on prairie chicken PBMC

The lack of reagents for most avian species makes it difficult to evaluate lymphocyte populations. A panel of chicken specific MAb was used to determine their ability to react with PBMC from prairie chicken, which are a type of grouse. Several of these MAbs labeled the heterologous cells as shown by flow cytometry analyses (Table 6). The chicken specific mouse anti-chicken K55 and K1 and selected anti-chicken CD4 (CT4) and CD8 (3-298) specific MAbs could be used to identify these populations in prairie chicken PBMC.

As shown in Figure 14, the K55 MAb was used to identify cells with three distinct levels of expression as was observed above after labeling of domestic chicken PBMC. The dot plots of cells labeled with both K55 and either CD4 (CT4-FITC) or CD8 (3-298-FITC) specific MAbs are shown in Figures 14B and 14C. Similar to the chicken lymphocytes, the prairie chicken lymphocytes with T cell markers expressed intermediate levels of K55. Thus, this procedure could be applied to determining concentrations of specific T cell lymphocyte populations collected from the prairie chicken, as well as from the domestic chicken.

Table 6. Reactivity of anti-chicken monoclonal antibodies with hybrid (APC/GPC) prairie chicken peripheral blood leukocytes.

Specificity	MAb	Isotype	Reactivity[a]	Reference
CD8	11-39	IgG1	-	Luhtala et al. (1995)
	11-38	IgM	-	Luhtala et al. (1995)
	11-13	IgG1	-	Luhtala et al. (1995)
	11-9	IgM	-	Luhtala et al. (1995)
	9-8	IgG2b	-	Luhtala et al. (1995)
	3-110	IgG1	-	Luhtala (1998)
	3-157	IgG2b	-	Luhtala (1998)
	3-292	IgG2b	+	Luhtala (1998)
	3-298	IgG2b	+	Luhtala et al. (1997)
	EP72	IgG2b	+	Paramithiotis et al. (1991)
	CT8	IgG1	-	Chan et al. (1988)
CD4	2-6	IgG1	-	Chan et al. (1988)
	2-35	IgG2b	+/-	Luhtala et al. (1993)
	7-125	IgG1	+	Luhtala et al. (1993)
	10-3	IgG1	-	Luhtala et al. (1993)
	CT4	IgG1k	+	Luhtala et al. (1993)
CD3	CT3	IgG1	-	Chen et al. (1986)
Macrophage and Thrombocyte	K1	IgG2a	+	Kaspers et al. (1993)
Leukocyte Common Antigen	K55	IgG1	+	Chung et al. (1991)

[a] + = reactivity (>3.00%), +/- = some reactivity (1.01-2.99%), - = no reactivity (<1.00%)
[b] nd = not done

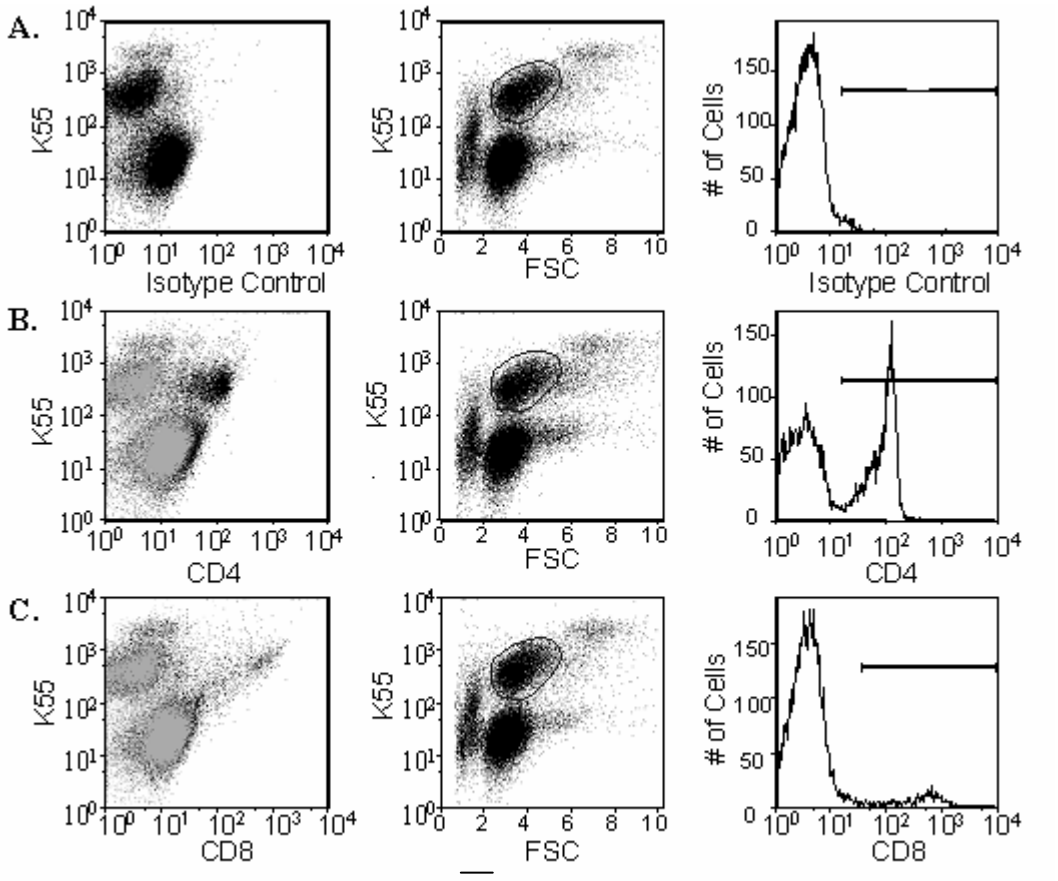


Figure 14. Dot plot of prairie chicken PBMC dual labeled with K55 MAb and lymphocyte markers. PBMC were labeled with an irrelevant antibody control (A), CD4 (B) specific and CD8 (C) specific MAbs. Columns are designated in Figure 2. Values on the x axis in the middle column represent PMT voltages times 100.

DISCUSSION

This study describes a method of distinguishing avian $CD4^+$ and $CD8^+$ lymphocyte populations in PBMC with the exclusion of thrombocytes. Separation of avian PBMC from whole blood with density gradient centrifugation resulted in a population of cells that otherwise would include up to 70% thrombocytes (personal observations), (Traill, 1983). Flow cytometric analyses of lymphocytes using labeled specific MAbs results in an overall low percentage of positive cells. Although not labeled, the majority of the cells observed are usually nucleated thrombocytes. Minor fluctuations in the percentage of thrombocytes can result in dramatic changes in the overall calculated percentages of B and T lymphocyte populations and subpopulations. By using K55 antibody to gate specifically on the lymphocyte populations, cells carrying the Bu-1^+, $CD3^+$, $CD4^+$ or $CD8^+$ phenotypes can be more reliably determined. Therefore, fluctuations in the concentrations of distinct phenotypes, that might indicate cellular immune stimulation or immunosuppression of these cells, can be accurately determined.

Whereas the K55 MAb segregated the leukocytes into distinct populations, the K1 and lymphocyte markers provided a mechanism to characterize these three populations. A large population of unlabeled leukocytes described by Chung et al. (1991) correlated with the low intensity labeled population in these studies. The K1 antibody reportedly identified an antigen present on the surface of both macrophages and thrombocytes (Kaspers, Lillehoj, and Lillehoj, 1993). Flow cytometric analyses of K55,

also labeled with K1, indicated that cells expressing low levels of K55 were expressing K1. Therefore, based on the relatively large percentage of the overall cell population that these K1 expressing cells represent, the K55-low cells consisted primarily, if not exclusively, of thrombocytes. The location of monocytes within the K55 subpopulations was more uncertain. K55 has been reported to associate strongly with monocytes, even more so than with lymphocytes (Chung, Lillehoj, and Jenkins, 1991). It is possible that monocytes segregate with thrombocytes in the K55 low fluorescence group. This strong association would presumably group monocytes in the K55 high fluorescence population. Yet, dual-labeling with both K55 and K1 did not detect K1 expressing cells in the K55-high fluorescence population. Further investigation of this K55 high fluorescence population is required in order to conclusively correlate the cellular composition of this group with monocytes and macrophages.

Because most avian reagents are only specific for poultry cell markers, it is difficult to evaluate the cellular immune status of other avian species, such as the endangered Attwater's prairie chicken. With the identification of mouse anti-chicken leukocyte MAbs that cross-react with the prairie chicken cells, this methodology was also applied to a second avian species. Although a number of MAbs specific for chickens did not react with the prairie chicken leukocytes, several were functional and could be reliably used for evaluations that provided dot plots, similar to those observed with the analyses of domestic chicken PBMC. The use of this procedure, using cross-reacting antibodies, allowed for the determination of lymphocyte evaluations of PBMC in a species of grouse.

CHAPTER V

FLOW CYTOMETRIC ANALYSIS OF REV INFECTION IN $CD4^+$ AND $CD8^+$ T CELLS

INTRODUCTION

Reticuloendotheliosis viruses (REV) are a group of similar viruses with a shared serotype (Chen et al., 1987). Disease caused by REV typically includes a runting syndrome combined with development of chronic neoplasia. Drew et. al. (1998) reported the first incidence of REV in a captive population of two subspecies of *Tympanachus cupido*, including both greater prairie chickens (*T. c. pinnatus*) and endangered Attwater's prairie chickens (*T. c. attwateri*), describing lymphocytic-histiocytic proliferative and infiltrative lesions on the head, legs, and feet. Subsequent infections within the flock resulted in multiple neoplastic lesions found in internal organs, primarily the liver and spleen. REV infection has become a significant problem for captive breeding facilities, as infected birds must be removed from breeding populations to eliminate transmission of the virus. Loss of birds in these populations hinders efforts in reestablishing numbers of this endangered species for release into the wild. Eliminating REV in captive populations of prairie chickens is likely critical to their survival as a species.

REV infection in several different avian species including chickens (Witter and Crittenden, 1979; Witter, Smith, and Crittenden, 1981), turkeys (Dren et al., 1988b; McDougall, 1980), and prairie chickens (Drew et al., 1998) is associated with the

development of neoplasia. Chronic neoplastic disease seen in chickens has been described as consisting of two types – bursal and nonbursal lymphomas (Witter, 2003). Bursal lymphomas are characterized by neoplasias commonly found in the liver, spleen, and bursa. Other organs are typically affected but with lower frequencies in these studies. Antibodies recognizing B cell antigens have been shown to label tumors, whereas T cell antigens were not present (Nazerian et al., 1982). Nonbursal lymphomas, have been described in experimentally REV infected chickens by Witter et.al. (1986), affecting the liver, spleen, heart, and thymus, but not the bursa. Analysis of tumor cells by antibody labeling revealed the presence of T cell antigens (Cooper, 1991). B cell antigens were absent in these tumors. Further investigation of non-bursal lymphomas from REV infected chickens identified the presence of the $CD8^+$ antigen, commonly found on cytotoxic T cell populations (Chan et al., 1988) in the most of the tumors studied (Cooper, 1991). CD4 antigen, a T helper cell marker (Chan et al., 1988), was also found in these tumors but at a lower frequency (Cooper, 1991).

Understanding the mechanisms of REV infection are critical for designing strategies for vaccine development to provide protection against the virus. Although neoplastic tissues resulting from REV infection have been characterized as having both B and T cell origins, the target cells of infection have not been identified. In this chapter, $CD4^+$ and $CD8^+$ T cells from REV infected prairie chickens were analyzed for support of REV infection.

MATERIALS AND METHODS

Birds, blood collection, and PBMC isolation

REV naturally infected and uninfected Attwater's prairie chickens were housed by the Small Upland-bird Research Facility (SURF) located at Texas A&M University, College Station, Texas. Infection was determined by nested PCR amplification of the polymerase in genomic DNA purified from peripheral blood mononuclear cells (PBMC) as described in Chapter 6.

Blood was collected from prairie chickens by jugular venipuncture. PBMC were separated from blood diluted with Alsever's solution using Histopaque-1077® according to manufacturer's instructions (Sigma Diagnostics, St. Louis, MO), with the exception of the addition of bovine serum albumen (BSA) to the wash buffer (Li et al., 2000). Briefly, at room temperature, 5 ml Histopaque®-1077 was overlaid with the blood/Alsever's mixture and centrifuged for 30 minutes at 400 x g at room temperature. Following centrifugation, the opaque interface was collected and washed twice at 4°C with phosphate buffered saline containing 0.2% bovine serum albumin and 0.2% sodium azide (PBA) and centrifuged for 10 minutes at 250 x g and 4°C (Li et al., 2000). Cell numbers were calculated and the cell concentration adjusted to a 1 x 10^6 cells/ml.

REV infection of chicken embryo fibroblasts (CEF)

CEF were cultured in 75 cm^2 flasks at 37° C with 5% CO_2 in 10 ml Dulbeco's minimal essential media (DMEM) with 10% fetal bovine serum until 80% confluent. Media was removed and cells were washed with 10 ml sterile phosphate buffered saline (PBS) containing Ca^{2+} and Mg^{2+}. Following removal of the PBS, cells were incubated with REV at a concentration of $5x10^4$ $TCID_{50}$ in 1 ml DMEM for 1 hour at 37°C. Nine ml of DMEM with 2% FBS were subsequently added to the flask and infected cells were cultured for 2 days at 37°C. Infected cells were then collected by trypsinization and washed twice with 10 ml of PBA prior to labeling for flow cytometric analysis.

Flow cytometry

All cell incubations and cell washes were done at 4°C. Prior to labeling with cell antigen specific MAb, nonspecific binding by cellular Fc receptors was blocked with normal sera (Li et al, 2000). A final concentration of 2mg/ml of normal goat IgG (Sigma Diagnostics, St. Louis, MO) was incubated with 1 x 10^6 cells/ml of PBMC for 10 minutes at 4^o C. Aliquots of 50 µl of 10^6 cells/ml suspension were incubated with each antibody diluted in PBA. Cells were double labeled with K55 and CD4 or CD8 directly labeled antibodies. Biotinylated K55 (diluted 1:100) and CD4 or CD8 (diluted 1:50) were added simultaneously and incubated for 30 minutes on ice. Cells were then washed with PBA. Streptavidin bound to R-phycoerythrin Cy5 (0.2 mg/ml stock) was

added to the cells to bind the biotinylated K55 at a dilution of 1:75. Cells were again incubated for 30 minutes and washed twice with PBA before resuspending in 200 µl 1% paraformaldehyde and incubating for 1 hour at 4^{o} C. Following paraformaldehyde fixation, cells were washed twice with PBA, resuspended in 200 µl of PBA, and stored at 4^{o}C. Controls were included for secondary antibody and normal primary sera.

Intracellular labeling was conducted using the Fix & Perm Cell Permeabilization Kit (Caltag Laboratories, Burlingame, CA). Cells labeled for both extracellular and intracellular antigens were labeled with extracellular antibodies as described above, before permeabilizing according to manufacturer's instructions and labeling cells with a 1:100 dilution of rabbit anti gag antibody.

Flow cytometric analyses was performed within 24 to 48 hours of sample processing with a FACSCalibur^{TM} (Becton Dickinson, San Jose, CA) by the Core Flow Cytometry Facility at Texas A&M University. Percentages of $CD4^{+}$ and $CD8^{+}$ T cells were based on the number of lymphocytes labeled in the K55 – intermediate positive population as previously described (Chapter IV).

RESULTS

REV infected Attwater's prairie chickens have decreased $CD4^+$ T cell percentages

$CD4^+$ T cell percentages were compared in 4 REV infected and 5 REV negative Attwater's prairie chickens (Fig. 15A). $CD4^+$ T cell percentages in the infected birds were significantly lower than those in uninfected birds. $CD8^+$ T cell percentages in both groups were also compared (Fig. 15B), however, no significant difference in percentages were noted between the two groups.

Both $CD4^+$ and $CD8^+$ T cells are infected with REV

Chicken embryo fibroblasts (CEF) infected by REV were used to collect stocks of the virus. In order to check for the ability of rabbit anti gag to bind the viral antigen in infected cells, intracellular staining methods and flow cytometric analyses were utilized. Both infected and uninfected CEF were permeabilized and labeled with rabbit anti gag conjugated to FITC. Fifteen percent of infected cells were positive for gag antigen as compared with 0% of uninfected cells (Fig. 16A). Both uninfected and infected cells were then examined for specificity of the antibody with gag antigen by first adding unlabeled antibody diluted at a 20 fold higher concentration than the working dilution to block all epitopes from labeling with the FITC conjugated antibody. Cells were subsequently incubated with labeled antibody at the working dilution of

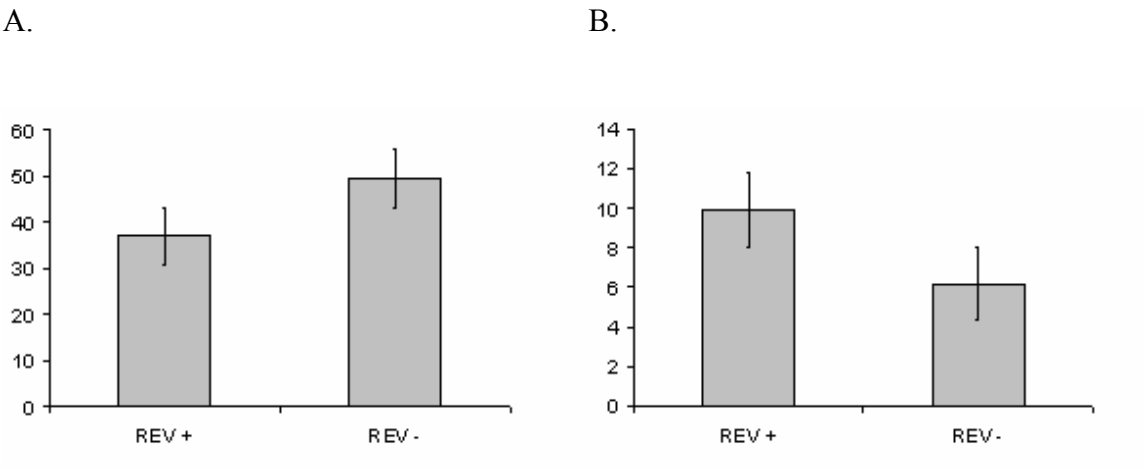
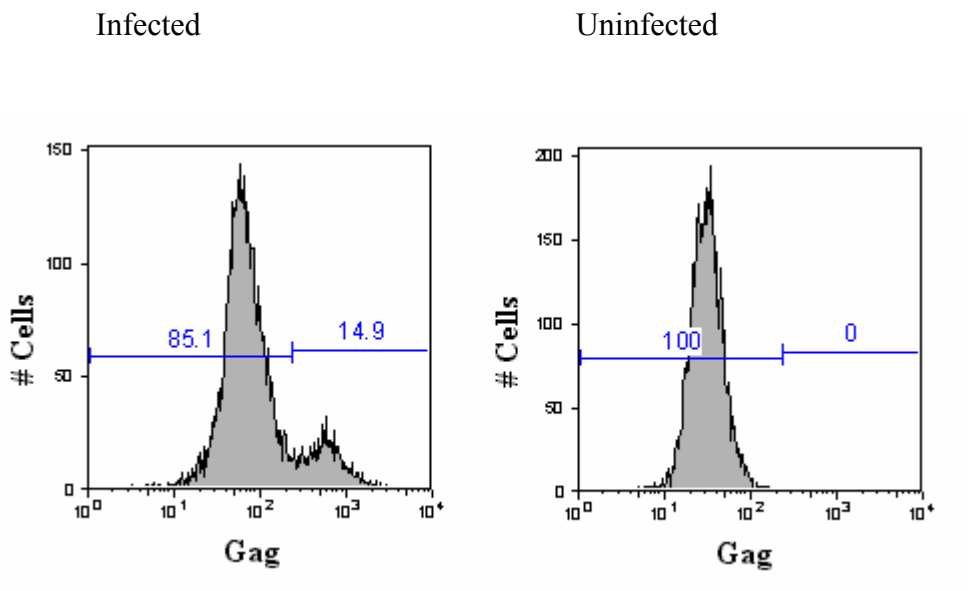


Figure 15. Comparison of $CD4^+$ (A) and $CD8^+$ (B) T cell percentages in REV infected and uninfected Attwater's prairie chickens.

A.



B.

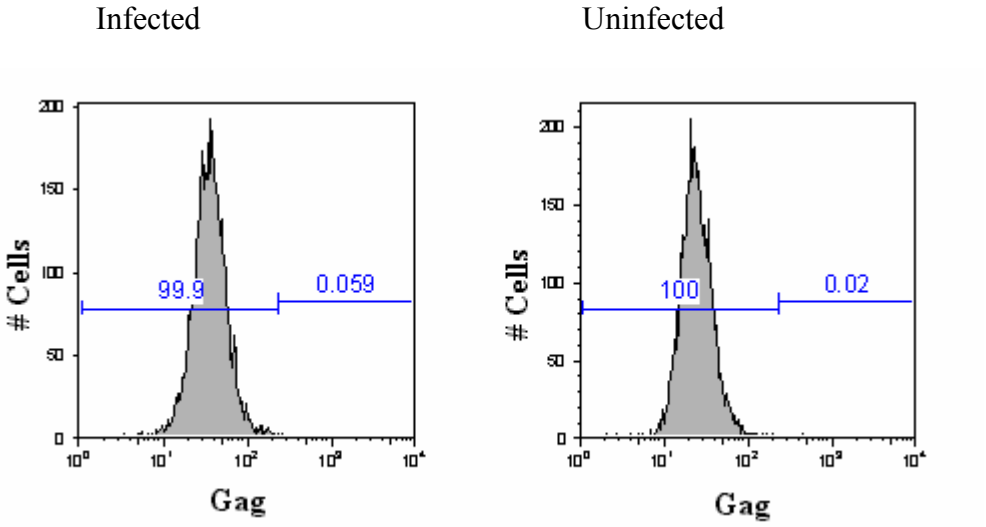


Figure 16. Histograms showing percentage of cells positive for gag antigen. A. Infected and uninfected cells labeled with polyclonal rabbit anti REV gag-FITC. B. Infected and uninfected cells labeled with polyclonal rabbit anti REV gag-FITC following blocking with unlabeled rabbit anti gag at a 10 fold higher concentration than the working dilution. C. Background cells labeled by rabbit IgG – FITC.

C.

Infected Uninfected

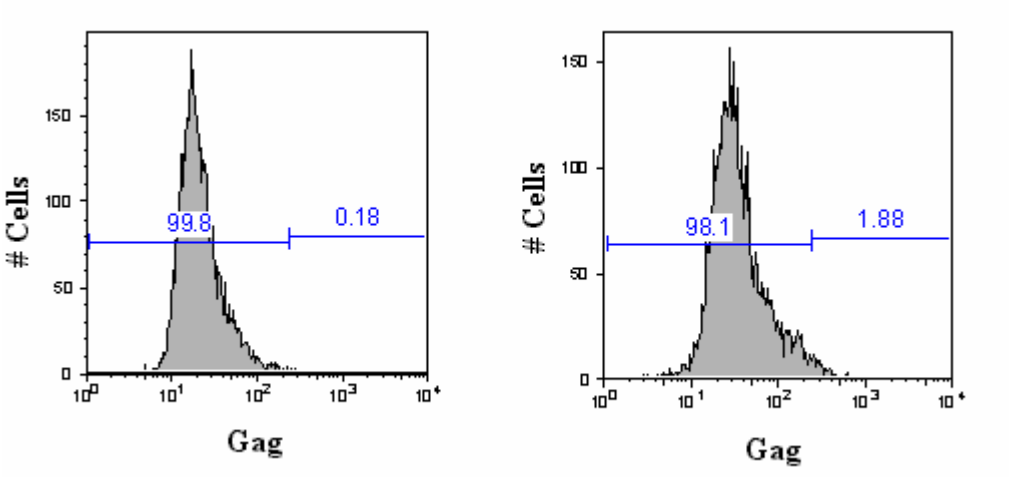


Figure 16. Continued

1:100. Both uninfected and infected cells show negligible labeling with the FITC conjugated rabbit anti-gag antibody indicating the antibody is specifically labeling the antigen (Fig. 16B). No nonspecific binding was seen with rabbit IgG – FITC in either infected or uninfected cells, further confirming that there is no nonspecific binding under these conditions (Fig. 16C).

Changes in $CD4^+$ and $CD8^+$ T cell percentages of REV infected prairie chickens, along with previous reports of nonbursal lymphomas resulting from REV infection, indicated the potential infection of these lymphocyte subpopulations with REV. Permeabilization and flow cytometric analyses were used on PBMC collected from both uninfected and infected Attwater's prairie chickens. Cells were labeled with K55 specific leukocyte monoclonal antibody, CD4 or CD8 specific monoclonal antibodies, and rabbit anti gag polyclonal antibody. Figure 17A is a contour plot of K55 positive cells from an REV infected Attwater's prairie chicken labeled with both CD4 and REV gag specific antibodies. K55 allows for analysis of the lymphocyte population with the exclusion of thrombocytes. A population of $CD4^+$ cells that are also positive for REV represented 12.1% of the total lymphocyte population and 77.7% of $CD4^+$ cells. A large percentage were also $CD4^-$, REV gag positive lymphocytes (53.6%). Negligible background staining was observed by the REV gag antibody in cells from the uninfected bird as only 1.6% of the total lymphocyte population were labeled (Fig 17B), further confirming the specificity of the antibody for infected cells.

A. B.

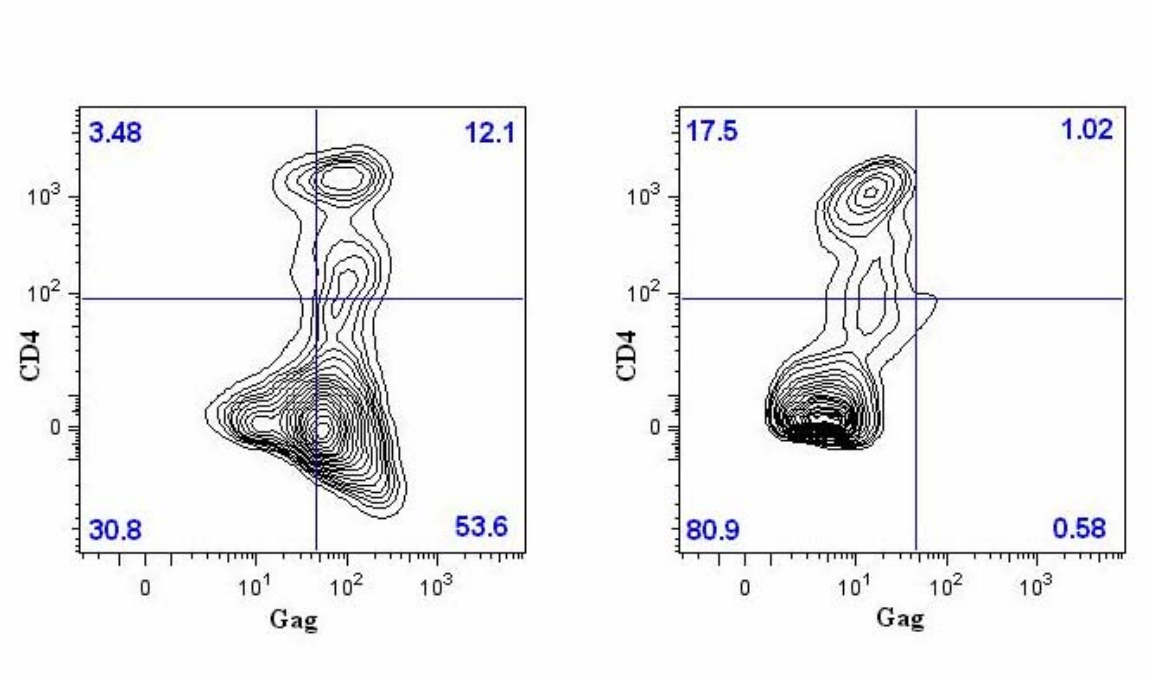


Figure 17. K55-intermediate positive Attwater's prairie chicken PBMC labeled with CD4 and REV gag antibodies. PBMC from both REV infected (A) and uninfected (B) birds were analyzed. Upper right quadrant represents cells positive for both CD4 and REV gag.

Infection of $CD8^+$ T cell with REV was determined using PBMC from REV infected (Fig 18A) and uninfected Attwater's prairie chickens (Fig. 18B). A large population of $CD8^+$ T cells positive for REV gag represents 8.75% of the total lymphocyte population and 82.8% of the $CD8^+$ T cells. As with the CD4 labeled cells, a large population of $CD8^-$, REV positive cells representing 55.7% of the total lymphocyte population were identified.

Labeling by the REV gag antibody was consistent in the infected samples with 65.7% (Fig. 17A) and 64.5% (Fig. 18A) of all lymphocytes expressing the gag antigen in each group. Infected $CD4^+$ and $CD8^+$ T cells combined only accounted for 21% of the total lymphocyte population leaving approximately 44% of the remaining lymphocyte population labeled by the REV gag antibody unaccounted for. Background staining of only 1.6% (Fig. 17B) and 0.95% (Fig. 18B) of all lymphocytes were labeled in the uninfected controls.

A population of cells expressing high levels of K55, presumed to be monocytes, were also analyzed for their expression of REV gag antigen. A vast majority (93.3%) were labeled by the REV gag antibody (Fig. 19A). K55 – low positive cells consisting mainly of thrombocytes (Chapter IV) were examined for REV infection as well. Figure 19B shows almost 90% of thrombocytes were positive for the REV gag antigen.

A. B.

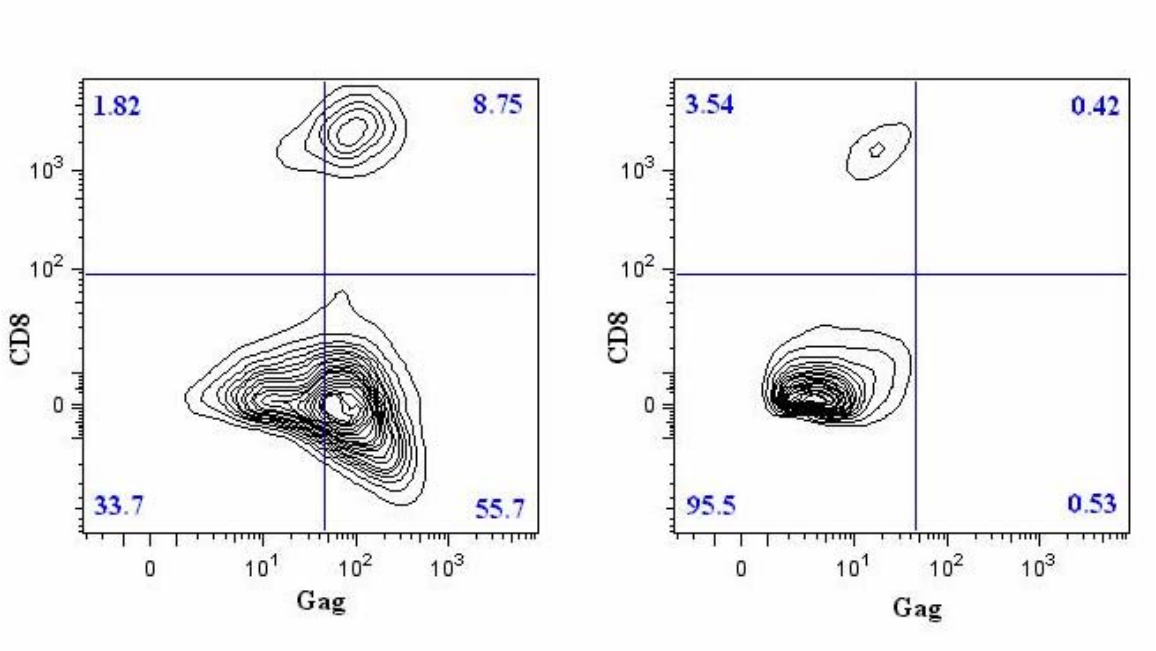


Figure 18. K55-intermediate positive Attwater's prairie chicken PBMC labeled with CD8 and REV gag antibodies. PBMC from both REV infected (A) and uninfected (B) birds were analyzed. Upper right quadrant represents cells positive for both CD4 and REV gag.

A. B.

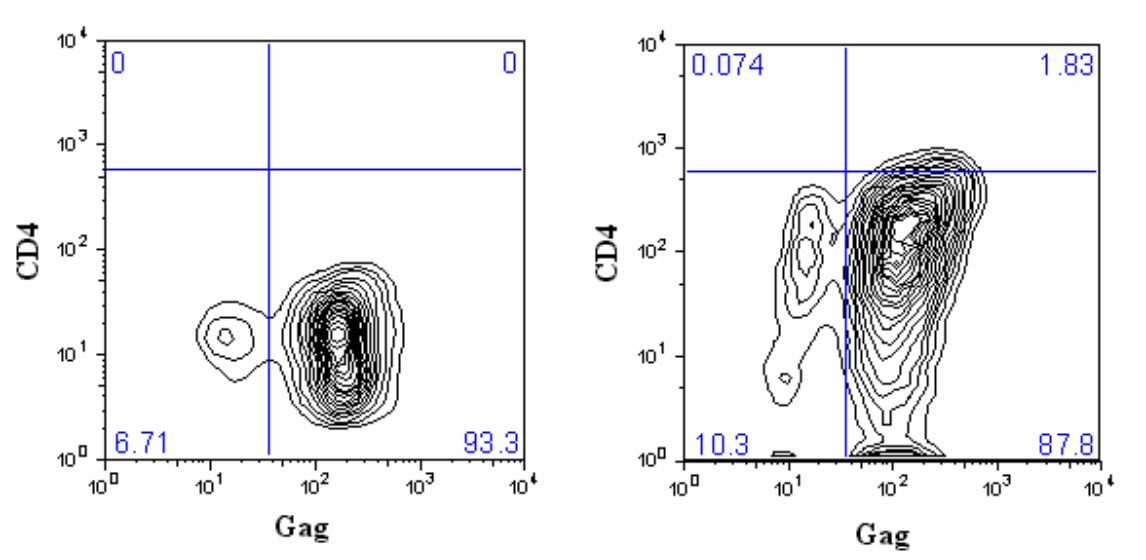


Figure 19. K55-high and K55-low positive PBMC labeled with REV gag antibodies. A. K55-high cells labeled with CD4 and REV gag antibodies. Lower right quadrant represents cells positive for REV gag. B. K55 – low cells labeled with CD4 and REV gag antibodies. Lower right quadrant represents cells positive for REV gag.

DISCUSSION

The total percentage of lymphocytes in the peripheral blood labeled by REV gag was relatively high with 75%-79% of all lymphocytes in PBMC expressed the gag antigen. As REV is known to cause lymphomas in infected birds, this high percentage may be the result of a large number of transformed cells. Cooper et al. (1991) report the frequent presence of $CD8^+$ T cells in non-bursal lymphoma tumors as well as the presence, to a lesser degree, of $CD4^+$ T cells. The data presented here indicated that a majority of both $CD4^+$ and $CD8^+$ T cells in PBMC were infected with REV.

However, the total percentage of lymphocytes infected represents a larger population than is typically accounted for by only $CD4^+$ and $CD8^+$ T cells, thus suggesting other lymphocyte subpopulations, such as B cells, may be infected with REV. Nazerian et al. (1982) have shown B cell antigens to be present on the surface of tumors in chickens with chronic bursal lymphomas. The absence of a B cell marker recognizing prairie chicken antigen at the time of this study, prevented analysis of REV infection of B cells. Although an antibody specific for the prairie chicken IgY has been developed, it was incapable of identifying an exclusive population as all lymphocytes were nonspecifically labeled with this antibody (data not shown). Antibodies specific for a B

cell antigen in prairie chickens have yet to be identified, but are required for further analysis of this lymphocyte subpopulation.

A population of K55 cells staining with a higher intensity than lymphocytes also stained positive with REV gag antigen. Although the identity of this population remains uncertain, K55 has been reported to have a stronger association with monocytes than with lymphocytes (Chung, Lillehoj, and Jenkins, 1991). This association may be indicative of a majority of monocytes infected with REV. Prairie chicken monocyte markers are also needed for further confirmation of potential infection with REV.

A majority of K55 - low positive cells, consisting mostly of thrombocytes, also stained with the anti-REV gag antibody. The absence of a population of cells remaining unlabeled by REV, in this study, suggests the need for further investigation of REV positive populations. Immunofluorescent assays (IFA) for visual detection of cell-specific and REV antigens may be necessary. Additionally, positive selection of lymphocyte subpopulations and monocytes, followed by PCR amplification of REV genes in genomic DNA would be useful in confirming the presence of REV infection.

CHAPTER VI

EXPERIMENTAL INFECTION OF ATTWATER'S/GREATER PRAIRIE CHICKEN HYBRIDS WITH THE RETICULOENDOTHELIOSIS VIRUS

Reticuloendotheliosis virus (REV), a common pathogen of poultry has been associated with runting and neoplasia in an endangered subspecies of grouse, the Attwater's prairie chicken (*Tympanachus cupido attwateri*), a subspecies of *Tympanachus cupido*. The pathogenesis of REV infection was examined in experimentally infected prairie chickens. Three groups of 4 Attwater's/greater hybrid prairie chickens each were infected, intravenously, with varying doses (TCID50 200, 1000 and 5000) of REV isolated from a prairie chicken. A fourth group of 4 birds were not infected. Blood was collected prior to infection, and at various times up to 37 weeks following infection. Peripheral blood mononuclear cells were examined for integrated proviral DNA by a single amplification PCR and nested PCR of a region within the pol gene. The nested PCR identified REV proviral DNA in all REV inoculated birds by 2 weeks post infection (p.i.) and confirmed chronic infection throughout the study. With the exception of a bird that died from bacterial pneumonia 8 weeks (p.i.), neoplasia, resembling that seen in naturally occurring infections, was observed in all birds, even those receiving as little as 200 TCID50 of virus.

INTRODUCTION

The reticuloendotheliosis virus (REV) is a gammaretrovirus exhibiting a type C morphology. It has a simple genome encoding gag, protease, polymerase, and envelope genes. Several strains exist, including REV-T an oncogenic, replication defective virus (Hoelzer, Franklin, and Bose, 1979), REV- A, the replication competent helper virus associated with REV-T (Witter, Smith, and Crittenden, 1981), chick syncytial virus (CSV) (Cook, 1969), spleen necrosis virus (SNV) (Trager, 1959) and duck infectious anemia virus (DIAV) (Ludford, Purchase, and Cox, 1972). Naturally occurring infections are common in domestic chickens (Witter and Johnson, 1985) and turkeys (Dren et al., 1988b) and have been documented in other avian species, including ducks (Grimes and Purchase, 1973), peafowl (Miller et al., 1998), pheasants (Dren, 1983), geese (Dren, 1988a) and quail (Carlson, 1974). Many of these infections appear to result in minimal, and typically subclinical, disease. Birds experimentally infected with REV, develop an initial viremia that is controlled with the onset of an antibody response (Bagust, 1979; Witter et al., 1979). The antibody titers persist but gradually decline over time.

Infection with REV, under the appropriate circumstances, may result in significant morbidity and mortality. Infected poultry have been shown to have reduced weight gain (Witter et al., 1979; Witter, Purchase, and Burgoyne, 1970), abnormal feather development (Tajima, Nunoya, and Otaki, 1977), and immunosuppression (Scofield and Bose, 1978; Witter et al., 1979; Witter, Smith, and Crittenden, 1981) resulting in an increased susceptibility to other infectious diseases. Natural and

experimental infections have also been documented to result in the development of neoplasia (Ratnamohan et al., 1980; Solomon, Witter, and Nazerian, 1976; Witter, Smith, and Crittenden, 1981; Witter, 1979). The defective strain T, carrying the v-rel oncogene (Sevoian, 1964c), typically induced tumors developing within several weeks following infection. Infection of birds with nondefective REV strains also results in tumors, however, incubation times can range between 4-30 weeks following infection depending on the virus strain and the species and age of the bird infected (Witter, 2003). Although lymphoid and reticuloendothelial sarcomas are most commonly reported, mxyosarcomas, fibrosarcomas, and renal adenocarcinoms may also be observed (Witter, Smith, and Crittenden, 1981). Tumors that contain cells resembling lymphocytes or lymphoblasts have been shown to be of B cell, T cell, or myeloid origin (Cooper, 1991; Nazerian et al., 1982; Witter, Smith, and Crittenden, 1981). The origin of the cells resembling reticuloendothelial cells has not been determined. Tissue distribution of the tumors will vary according to the REV strain and the strain or species of bird infected. Tumors are typically found in the liver and spleen (Sevoian, 1964c; Theilen, Zeigel, and Twiehaus, 1966; Witter, Smith, and Crittenden, 1981). Other organs that are variably involved include the kidney, pancreas, heart, esophagus, proventriculus, intestines, skin, brain, and peripheral nerves (Grimes, 1979; Sevoian, 1964c; Theilen, Zeigel, and Twiehaus, 1966; Witter and Crittenden, 1979; Witter, Smith, and Crittenden, 1981).

Drew et. al. (Drew et al., 1998) reported cases of REV infection and associated disease in two subspecies of prairie chicken (*Tympanachus cupido*) - the Attwater's prairie chicken (APC; *Tympanachus cupido attwateri*) and the greater prairie chicken

(*Tympanicus cupido pinnatus*). The Attwater's prairie chicken is highly endangered and its numbers in the wild have been reduced to less than 200 birds. Neoplastic lesions consisting of multifocal lymphoreticular proliferations consistent with those induced by REV in other species of birds were recognized in both wild caught and captive, naturally infected APC's. Subsequently, infected birds have been identified in five other captive breeding facilities, and infected birds in the wild have been documented (Bohls 2005, unpublished). Currently this disease is controlled in captive populations by culling birds that test positive by PCR amplification of a region of the REV pol gene. Removing infected birds from the population is considered essential as they may serve as a source for horizontal transmission, are likely to develop neoplasia, and cannot be used for breeding because of the concern for vertical transmission. However, culling can significantly reduce the number of birds that can be released as part of a reintroduction program and could eventually result in the loss of genetic variability within the species.

The pathogenesis of REV in prairie chickens has not been experimentally demonstrated. In this report, the infectivity and pathogenicity of three doses of REV given, intravenously, to adult Attwater's and greater prairie chicken hybrids are compared. Additionally, a new, more sensitive PCR detection method of REV was developed for early detection of REV infection.

MATERIALS AND METHODS

Experimental birds

Twelve female Attwater's/greater hybrid prairie chickens were randomly divided into 3 treatment groups of 4 birds each. Treatment groups were housed in a single isolation building with negative air flow. Groups were separated by 1.2 meter high painted wood panels. Sealant was applied between panels and the floor to prevent exchange of bedding material. Plastic netting was stretched above the walls to add another meter of height. A fourth group of uninfected male greater/Attwater's hybrids that served as controls were housed separately outdoors in a 143 m^2 triangular pen surrounded on the top and sides with nylon 2.5-cm mesh. A tin roof was extended out 3 m in one corner to provide shelter. All birds were fed Mazuri game bird feed. The control birds were not euthanized at the end of the experiment, however, 3 male and 1 female Attwater's/greater hybrids that were REV negative died tragically during transport and were necropsied as histopathological controls.

Experimental infection and monitoring

An REV strain had been isolated from a naturally infected Attwater's prairie chicken (isolate PC-R92) testing positive for REV by PCR amplification of the LTR (Aly, 1993) (TVMDL). Buffy coats were collected from 0.5 ml of blood and were

cultured in the presence of chicken embryo fibroblasts (CEF) in Dulbeco's minimal essential media (DMEM) with 2% fetal bovine serum (FBS). Supernatants were removed from cells and stored at -80° C. Viral stocks were passaged two additional times on CEF and $TCID_{50}$ concentrations were determined by REV gag antigen ELISA (Bohls, unpublished data).

Two weeks prior to inoculation with REV, all birds were bled and tested for REV by nested PCR as described. Three groups of Attwater's/greater prairie chicken hybrids were infected with REV at varying doses [$TCID_{50}$ 200 (Group 1), 1000 (Group 2) and 5000 (Group 3)]. Virus was diluted in 0.5 ml sterile PBS and given intravenously into the right jugular vein. The control group received a sham inoculation of PBS only. Birds were monitored daily for signs of illness. Birds were euthanized at week 21 (Group 1) and week 37 (Groups 2 and 3) by carbon dioxide asphyxiation. Original plans were to necropsy all birds at 20 weeks post infection. However, following necropsy of group 1 birds, gross neoplastic changes were not found. Therefore, the duration of the study was extended for the remaining 2 groups of infected birds to allow potential neoplastic development.

Collection of blood

Three ml of blood were collected via jugular venipuncture at 2 weeks pre infection and weeks 2, 4, 7, 10, 14, 19, 25, and 37 post infection. Blood was diluted 1:1 with Alsever's solution and under-laid with 2 ml of Histopaque (Sigma, St. Louis, MO).

Cells were spun at 400 x gravity for 30 minutes. Peripheral blood mononuclear cells (PBMC) were collected from the suspended layer and washed twice in 10 ml PBS with 0.02% BSA and 0.02% sodium azide (PBA).

PCR

Genomic DNA was extracted from PBMC from 0.5 ml of blood using the GenElute Mammalian Genomic DNA Miniprep kit (Sigma, St. Louis, MO) and stored at -20 °C. Two µl of genomic DNA template were added to a 50 microliter (µl) reaction containing 36.75 µl water, 5 µl 10X Mg free reaction buffer (Promega, Madison, WI), 3 µl 10 millimolar (mM) $MgCl_2$, 1 µl 10 mM dNTP mix, 1 µl (10 pmol) of forward primer, 1 µl (10 pmol) reverse primer and 0.25 µl Taq polymerase (Promega). Samples where held at 95º C for 5 minutes, then cycled at 95º C for 30 seconds, 55º C for 30 seconds and 72º C for 2 minutes for 40 cycles. Samples were then held at 72º C for 7 minutes and then stored at 4º C. Primers used for first step amplification PCR were REVR3559 (TGCCACCCGAGACTTACTCA) and REVR5306 (CCCAGCCCGAGAATGTGTCTAC) within the polymerase gene. Positive samples yielded a predicted 1,748 bp product. Primers were chosen using OLIGO 4.0 software.

Following the first step PCR amplification, positive samples yielded a 1,747 base pair (bp) product. Two µl of the product from the reaction following the first step amplification were diluted 1:50 in water. Two µl of this dilution were added to a second 50 µl reaction (prepared just as the first and run under the same cycle conditions).

Primers used for the second step amplification of the nested PCR reaction were REVF3559 and REVR4138 (CTGCCCGAAGGTAAGTTTAGAG). Following the second step amplification, positive samples yielded a predicted 869 bp product. Positive controls of genomic DNA extracted from REV infected DF-1 cells as well as negative controls containing no DNA were included in all reaction sets. Five µl of PCR products following both first and second step amplifications were electrophoresed on 1% agarose gels in tris-acetate buffer (TAE; 0.4 M tris-acetate, 0.001 M EDTA) with 0.5 µg/µl ethidium bromide. Bands resulting in either first or second step amplification reactions indicated samples positive for REV.

PCR Standards

A pcDNA 3.1/V5-His TOPO TA vector (Invitrogen, Carlsbad, CA) with an insert corresponding to nucleotides 3,559 to 5,306 on the genome of a prairie chicken REV isolate PC-R92 (Bohls et al, in preparation) was used to transform chemically competent TOP 10 cells following the manufacturer's protocol (Invitrogen, Carlsbad, CA). Plasmid was grown up in the bacteria and purified with a GenElute Plasmid Miniprep Kit (Sigma, St. Louis, MO). Plasmid concentration was determined by spectroscopy. Serial, 10-fold dilutions of plasmid, ranging from 10^{9} to 10^{-1} copies per microliter, were used to determine the sensitivity of the single step PCR and nested PCR as described above.

Histologic examination of tissues

Liver, kidney, spleen, lung, heart, esophagus, proventriculus, ventriculus, duodenum, pancreas, jejunum, muscle, brain, colon, cecae, and sciatic nerve were also collected from each bird and fixed in 10% neutral buffered formalin. Formalin-fixed tissues were paraffin-embedded and 4 μm sections were cut. The sections were stained with hemotoxylin and eosin.

RESULTS

Nested PCR detected REV provirus in all virus inoculated birds

A nested PCR with two rounds of amplification was developed for sensitive detection of viral infection. The sensitivity of the nested PCR was determined using limiting dilutions of a pcDNA 3.1 TOPO plasmid with an insert corresponding to a region within the pol ORF of the REV genome. Nested primers specific for the cloned region were used to amplify the region. Ten fold serial dilutions (1x 10^{9} to 1x 10^{-1} copies/reaction) of the stock plasmid were used as template. The lowest detectable concentration using a one step PCR was 1 X 10^{5} copies/ reaction (Fig. 20). Following nested PCR amplification, bands were visible for dilutions down to 1 X 10^{2} copies/ reaction. No bands were seen for any of the lower dilutions (10^{1} to 10^{-1}) or the negative control.

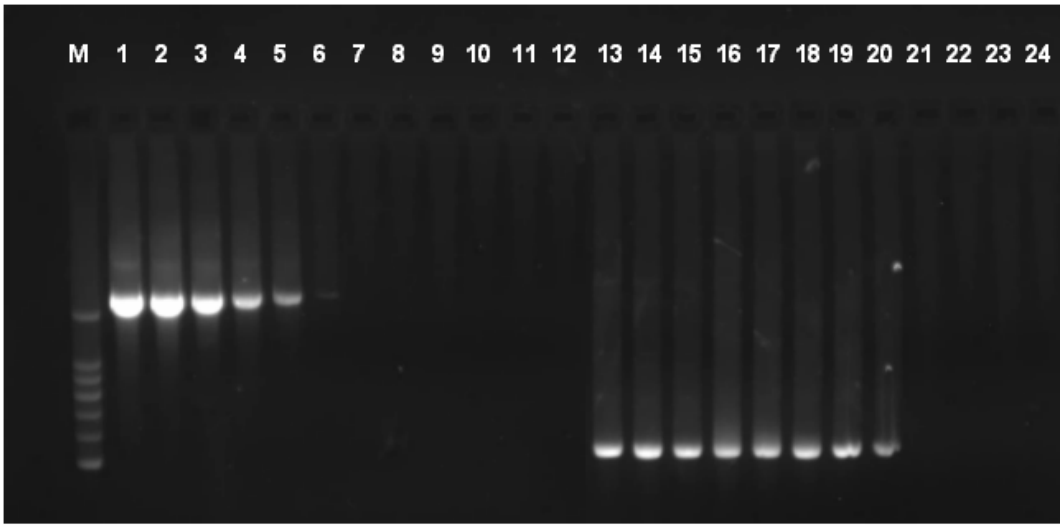


Figure 20. Agarose gel of PCR sensitivity. Lanes 1-12 represent single-step PCR reactions of 10 fold serial dilutions of template from 10^{9} copies/reaction (lane1) to 10^{-1} copies/ reaction (lane 11). Lanes 13-24 represent similar serial dilutions of template in a nested PCR reaction from 10^{9} copies/reaction (lane 13) to 10^{-1} copies/reaction (lane 23). Lane 12 and 24 represent the negative controls with no template.

Blood samples from all prairie chickens were examined for REV provirus by both single step amplification and the more sensitive nested PCR. The results are shown as the percent of birds in a group that were positive by each PCR amplification for the week of sample collection (Table 7).

Whereas, single step PCR amplification resulted in a variable percentage of positive tests throughout the duration of the study, the nested PCR protocol detected proviral REV in the PBMC of all birds regardless of dose by 2 weeks PI. Furthermore, nested PCR amplification of DNA from PBMC detected provirus in 100% of birds in all treatment groups at every time interval tested, after 2 weeks.

Neoplasia was found in birds infected with all 3 doses

A group 1 bird died spontaneously 8 weeks after infection. The death of this bird was attributed to an extensive local bacterial pneumonia. *Pseudomonas arugenosa* was grown in pure culture from the lung. Neoplastic lesions were not seen grossly nor histologically in this bird and it was not included in subsequent analysis. The remaining birds in group 1 were euthanized and necropsied 21 weeks after infection. A group 3 bird died 3 days later and was necropsied. A group 2 bird died 23 weeks after infection. The remaining group 2 and 3 birds were euthanized and necropsied 37 weeks after infection.

Table 7. Percentages of positive birds in each week post infection by nested PCR.

		2		4		7		10		14		19		26		37	
	PCR Assay[A]	SA	NT	SA	NT	SA	NT	SA	NT	SA	NT	SA	NT	SA	NT	SA	NT
Group #	Uninfected	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
	Group 1 $TCID_{50}$ 200	25	100	0	100	0	100	33	100	33	100	33	100	n/a	n/a	n/a	n/a
	Group 2 $TCID_{50}$ 1000	25	100	75	100	50	100	100	100	75	100	100	100	75	100	100	100
	Group 3 $TCID_{50}$ 5000	100	100	100	100	50	100	25	100	100	100	100	100	66	100	100	100

[A]SA = single amplification PCR; NT = nested PCR.

Gross neoplastic lesions were seen in the birds from group 2 and 3 that died spontaneously and in two group 3 birds that were euthanized. Lesions typically consisted of multifocal, raised, round to irregular, grey tan nodules that were randomly scattered within the liver and, in 2 birds, the spleen (Fig 21). Much of the normal liver was replaced by coalescing neoplastic foci in the two birds that died spontaneously and in one of the group 3 birds that was euthanized. Microscopic neoplastic lesions typical of REV infection were found in the liver and spleen of birds in both groups 2 and 3. However, although microscopic neoplastic lesions of the spleen were detected in 100% of the group 1 birds, no neoplastic liver lesions were detected in this group.

Neoplasia was found microscopically in all the experimentally infected birds, with the exception of the bird in group 1 that died with a bacterial pneumonia. Most neoplasms were composed of sheets of large round to polygonal and less commonly spindloid cells that had a large central round to oval nucleus with 0 to 2 nuclei, prominent clumps of heterochromatin were present in most nuclei. Surrounding each nucleus was a small to moderate rim of eosinophilic cytoplasm with the typical nuclear to cytoplasmic ratio being 2:1. Mitotic figures were common (5-10 per high powered field) (Fig. 22). A fine fibrovascular stroma was present in most neoplasms, however, fibroplasmia was locally prominent in a few of the neoplasms. In all tissues, neoplasms enlarged by both expansion and invasion causing compression and atrophy of adjacent tissues (Fig. 22).

Figure 21. Photograph of the liver of an Attwater's/greater prairie chicken hybrid that died 33 weeks after infection with 1000 TCIDs of reticuloendotheliosis virus. The liver contains multiple neoplastic nodules.

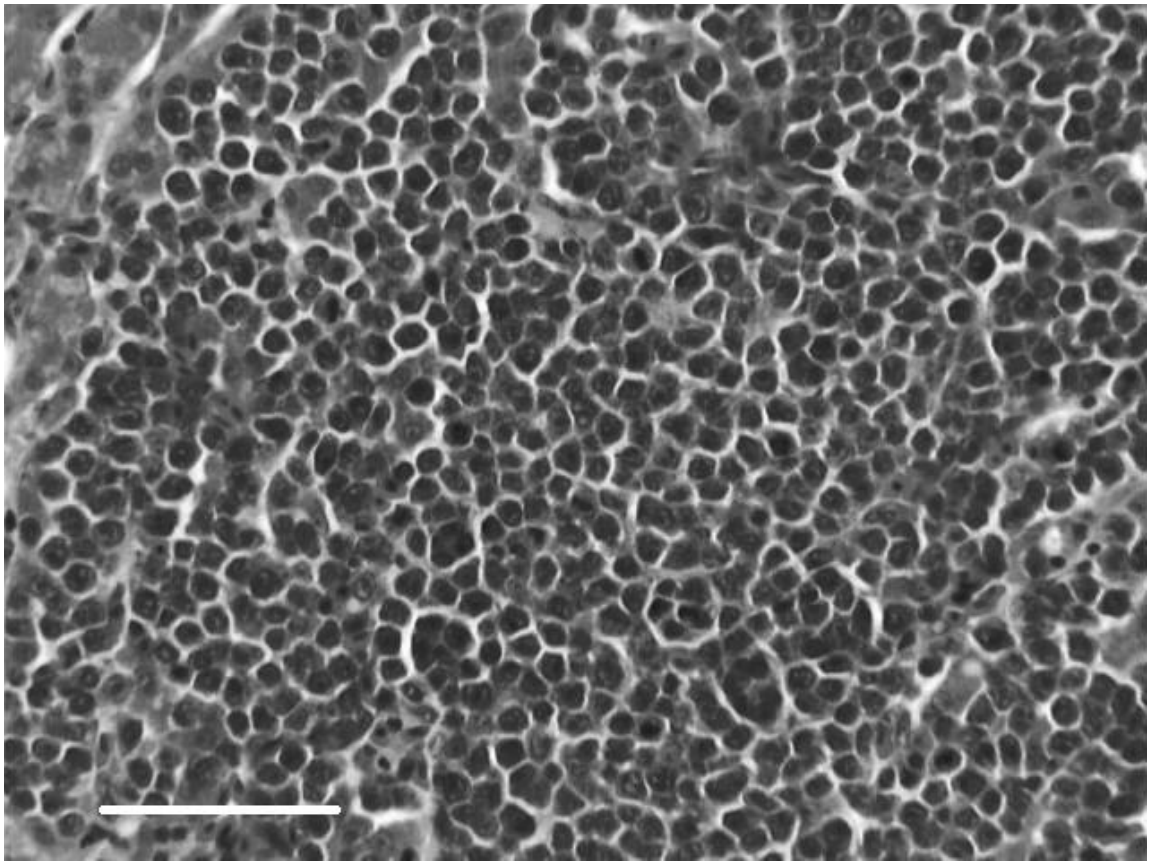


Figure 22. Photomicrograph (high mag.) of the liver of an Attwater's/greater prairie chicken hybrid that died 33 weeks after infection with 1000 TCIDs of reticuloendotheliosis virus. Note the characteristic sheets of reticuloendotheial-like neoplastic cells. Bar = 50 μm.

Neoplastic changes varied in extent between birds. Lesions in the group 1 birds were subtle and were predominately confined to neoplastic cells forming halos around the sheathed splenic arteroles (Fig. 23). A focal neoplastic lesion was also found in the lamina propria of the duodenum of one of these birds, two neoplastic foci were found in the kidney of a second bird, and a diffuse infiltration of the neoplastic cells was found in one section of the lamina propria of the jejunum from the third bird in group 1 (Table 8).

Three of the four group 2 birds had splenic lesions that were similar to those seen in the group 1 birds. Two of these three birds, however, had more extensive digestive lesions with multiple neoplastic foci present in all sections of the esophagus and one or two large foci at the esophageal-proventricular junction (Fig. 24; Table 8). All esophageal neoplasms developed in the loose connective tissue below the esophageal epithelium. Enlargement of these neoplasms resulted in compression of adjacent esophageal glands. The larger neoplasms invaded the muscular layer, but rarely would invade the epithelium. Ulceration of the esophageal mucosa was rare. The fourth bird in group 2 died spontaneously. Upon necropsy, it was observed that the spleen of this bird was largely replaced with neoplastic cells and the spleen had infarcted. The liver contained multiple neoplastic nodules, some of which were also partially infarcted.

Three of the four group 3 birds had macroscopic neoplastic lesions of the liver and spleen. All four birds had halos of periarteriolar neoplastic cells. In the three birds with advanced lesions, the sheets of neoplastic cells were often coalescing and nodules of neoplastic cells were common between the splenic sheathed arterioles. Multiple coalescing nodules of neoplastic cells replaced 50% to 90% of the liver in the three birds

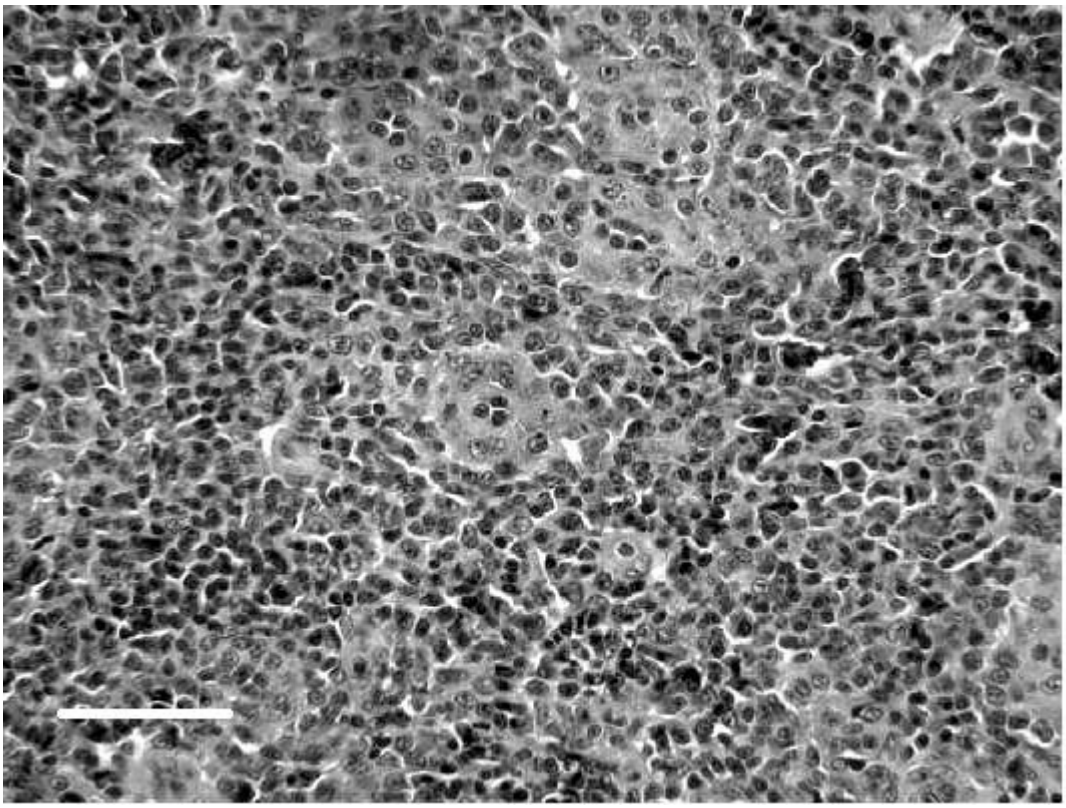


Figure 23. Photomicrograph of the spleen of an Attwater's/greater prairie chicken hybrid that died 33 weeks after infection with 1000 TCIDs of reticuloendotheliosis virus. The perivascular sheaths are surrounded by neoplastic cells. Bar = 50 μm.

Table 8. Distribution of neoplastic lesions in prairie chickens infected with 3 doses of reticuloendotheliosis virus.

Group	Spleen	Liver	Digestive	Other Tissues
1 ($TCID_{200}$) (n = 3)	100%	0%	33%	33%
2 ($TCID_{1000}$) (n = 4)	100% (25%)[A]	25% (25%)	75%	25%
3 ($TCID_{5000}$) (n = 4)	100% (100%)	75% (75%)	100%	50%
Control (n = 4)	0%	0%	0%	0%

[A] Numbers in parentheses are the percentage of birds in each group that had macroscopic neoplasms in each organ.

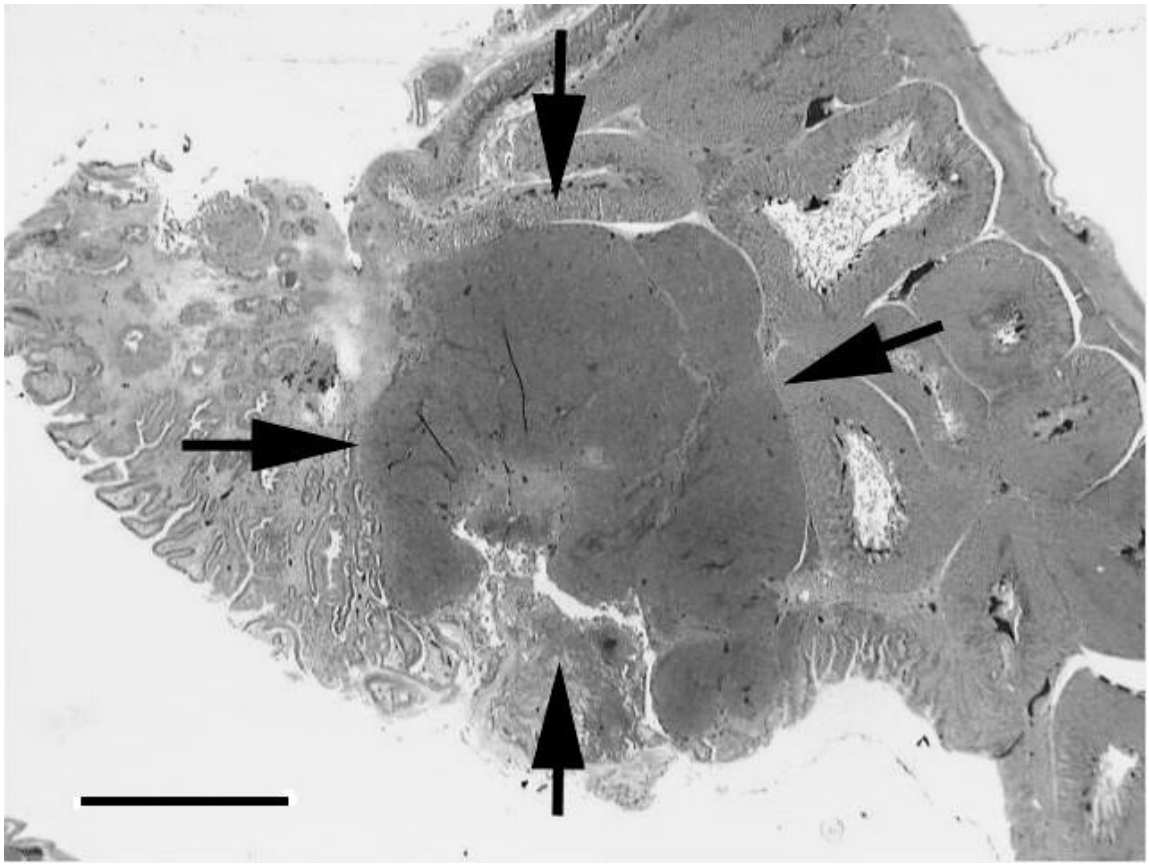


Figure 24. Photomicrograph of the esophageal junction with the proventriculus of an Attwater's/greater prairie chicken hybrid that died 33 weeks after infection with 1000 TCIDs of reticuloendotheliosis virus. A nodule of neoplastic cells compresses adjacent mucosal glands. Neoplasia of the esophagus and esophageal-proventricular junction was common in the experimentally infected birds. Bar = 1 mm.

with advanced lesions (Fig. 25). Neoplasia was not found in the liver of the fourth bird. Neoplasia of the esophagus and proventriculus was found in 3 of the 4 birds from group 3. Lesion descriptions were similar to those seen in group 2 birds only they tended to be more extensive. Autolysis precluded interpretation of the intestines of the bird that died spontaneously, but neoplastic disease of the lamina propria was present in the duodenum or jejunum and in the cecum of 2 of these 3 birds. Focal neoplastic nodules were found in the lung of 1 group 3 bird and the pancreas of another (Table 8).

Incidental necropsy findings in control and infected birds. *Capillaria* sp. were found in the cervical or thoracic esophagus of 75% of the infected birds and 100% of the control birds. There was no inflammation or a mild to moderate degree of inflammation associated with these parasites. Associated inflammation was predominately lymphoplasmacytic, but in some birds, heterophils were also seen in smaller numbers. The inflammatory lesions associated with the *Capillaria* sp. infection complicated the diagnosis of neoplastic change in the esophagus. All birds had pneumonoconiosis and a mild to moderate, locally extensive to diffuse, pneumoconiosis. This lesion was characterized by a bulbus thickening of the interatrial septae. Septae where distended with histocytic cells containing abundant fine brown dust-like particles. Most cells also contained fewer, up to 1 μm, black particles. Hypertrophy of the interatrial smooth muscle cells was prominent in two of the birds. Although these changes resulted in a reduction in the width of the affected atria, they did not appear to be sufficiently severe to interfere with airflow. Lesions were more advanced in the infected birds than the control birds.

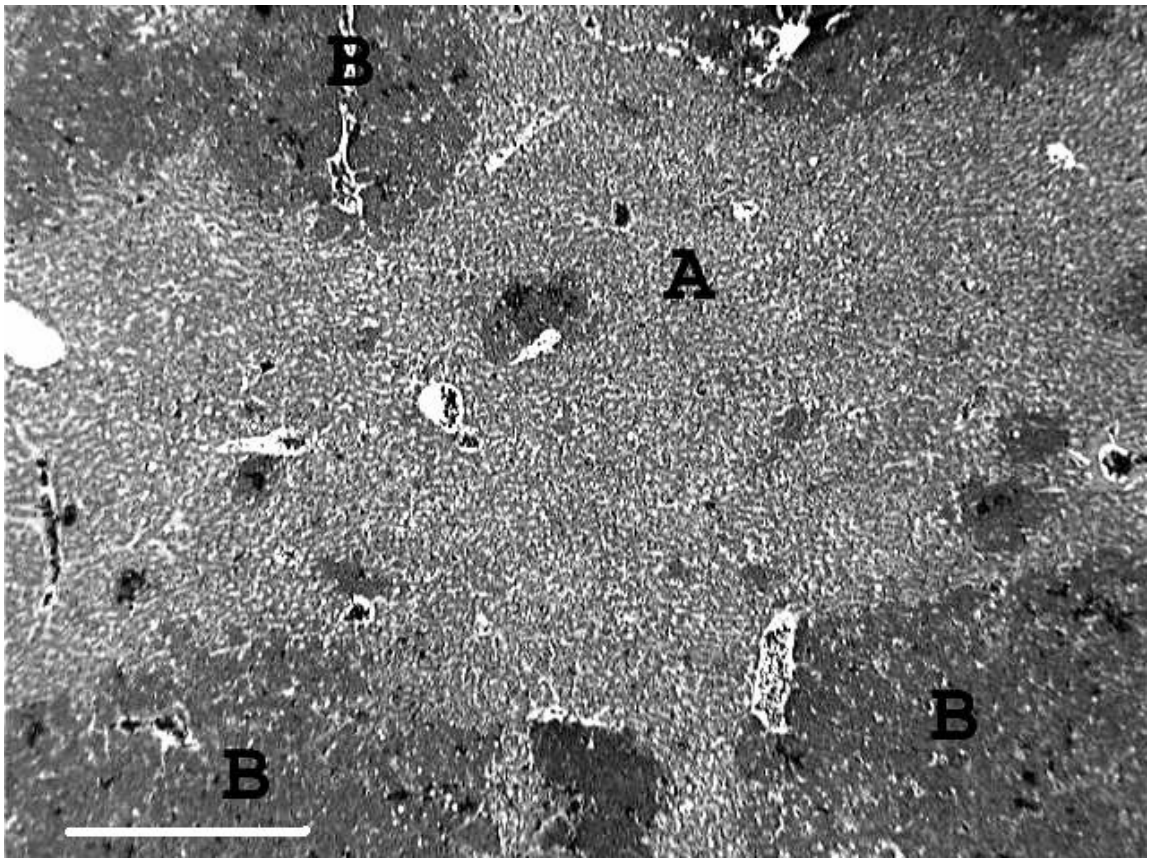


Figure 25. Photomicrograph (low mag.) of the liver of an Attwater's/greater prairie chicken hybrid that died 33 weeks after infection with 1000 TCIDs of reticuloendotheliosis virus. Normal liver (A) is being replaced by coalescing neoplastic foci (B). Bar = 0.5 mm.

A single bird in the high dose group was found to have a severe extensive chronic active septic granlomatous air sacculitis secondary to aspiration of food. Air sacs contained large masses of fibrin and blood that were surrounded by multinucleated giant cells, plasma cells, and lymphocytes. Intralesional plant material and colonies of bacteria were present. A single bird from the high dose treatment group had multifocal to locally extensive metastatic mineralization of the basement membranes of the proximal tubules of the kidneys. Mild to moderate metastatic mineralization of the adrenal was present in one control bird.

Two birds, one in group 1, and one in group 2, had a single, approximately 1 cm in diameter, mucosal papilloma of the proventricular mucosa. These papillomas had a moderately wide base and fibrovascular core that supported frond-like projections that were covered by a well organized and well differentiated tall columnar epithelium. The fibrovascular core in the papilloma in the low dosed bird was heavily infiltrated with heterophils, lymphocytes, and plasma cells. A small section of this papilloma was ulcerated. The papilloma in the moderate dose bird was adjacent to a submucosal neoplasm and its fibrovascular core contained lymphocytes, plasma cells and neoplastic cells. Similar papillomatous lesions were not seen in the control group.

DISCUSSION

REV infection in prairie chickens results in a lethal neoplastic disease (Drew et al., 1998) that has caused a major obstacle for captive breeding efforts to reestablish

numbers of this endangered species (personal observation). Genetic variability within the species is increasingly compromised as infected birds are removed from breeding populations in order to prevent the spread of the infection. The ultimate objective of the ongoing REV research in these birds is to develop a vaccine capable of protecting against infection. The first step toward this objective was to develop a model of REV infection that could be used subsequently to study the efficacy of a vaccination program.

Before conducting this study, it was necessary to develop a sensitive assay that could detect infection at its earliest stages. Previously, a PCR assay used in combination with a Southern blot has been described for use in detecting REV infected birds. This assay used primers that amplified a 291 base pair product in the long terminal repeat region of the REV genome (Aly, 1993). The assay was reported to be capable of detecting REV at 600 copies per reaction. Our goal was to develop a more sensitive and convenient test that could be used to screen large numbers of birds and presumably detect birds at an earlier stage of infection. The assay that we describe here was able to detect 100 target copies of the virus DNA making it 1,000 times more sensitive than a single amplification reaction and 6 times more sensitive than a single amplification reaction combined with a Southern blot. This assay had the added advantage that it detects the polymerase coding region giving a greater assurance that it is detecting whole virus genomes.

Using the single step PCR, fluctuations were observed in detectable viral loads in birds receiving any of the three doses of virus. However, all infected birds in the three treatment groups could be detected with the nested PCR assay by 2 weeks after

infection, and may have been detected earlier, had they been sampled earlier. Additionally, all infected birds tested positive with the nested test at every time point. Detection of REV provirus in single-step reactions is indicative of higher levels of provirus in the PMBC than in those samples which yielded no single-step reaction products. Changes in these detectable viral levels are most likely the result of fluctuating proviral levels in the PBMC of infected birds.

Even nondefective REVs that do not contain an oncogene, have been shown to naturally and experimentally induce neoplasms in chickens, pheasants, quail, turkeys, prairie chickens, ducks, and geese. Depending on the dose of the virus, the virus strain used, the species infected and the age of the birds at the time of infection, gross neoplastic lesions develop between 4 and 30 weeks post infection (Witter, 2003). Gross lesions in these birds can either be nodular or diffuse (Witter, Smith, and Crittenden, 1981; Witter, 1979). The spleen and liver are the two most commonly targeted organs, but tissue distribution of the tumors varies greatly between reports. In some outbreaks, tumors are found in one or more portions of the digestive system including the esophagus and crop, proventriculus, small intestine, and cecae. The heart, bone marrow, thymus, lung, pancreas, kidney, and thyroid have also been reported to contain tumors. Neoplastic cells are often lymphocytes and can be either of B cell or T cell origin (Cooper, 1991; Nazerian et al., 1982; Witter, Smith, and Crittenden, 1981). In other cases, infected cells more typically resemble reticuloendothelial cells or histiocytes.

The only published report of REV disease in prairie chickens describes lesions in both greater prairie chickens and the Attwater's subspecies. Some of the greater prairie chickens had neoplasms that were confined to the skin. These lesions were not seen in the Attwater's prairie chickens. Both greater and Attwater's prairie chickens had nodular lesions that were most commonly found in the liver and spleen. Additional affected organs included the lung, oral pharynx, and esophagus. Neoplastic cells in the skin, liver, and spleen of the greater prairie chickens were described as pleomorphic ranging from lymphoid to histocytic to fusiform. Neoplastic cells from the other organs were not described (Drew et al., 1998).

In this study, neoplasia was induced in all groups of birds irrespective of the infective dose given. Lesions were mildest in group 1 ($TCID_{200}$), but necropsies of these birds were done 4 months before the other 2 groups. The fact that 3 of 4 birds in group 2 ($TCID_{1000}$) had mild to moderate neoplastic disease as compared to group 3 ($TCID_{5000}$) where 3 of 4 had advanced disease suggests that there was a dose effect on the speed in which the induced neoplastic disease developed. The time course of the development of gross neoplastic lesions in the hybrid prairie chickens (21 to 37 weeks) is consistent with that reported in other experimentally infected poultry (Dren, 1988b; McDougall, 1978; Paul et al., 1976; Witter, Smith, and Crittenden, 1981). The neoplastic cells seen in these birds were fairly uniform from lesion to lesion and most closely resembled histiocytic cells. A more pleomorphic population of cells, as is reported in naturally infected prairie chickens was seen in only a few lesions (Drew et al., 1998). We did not

observe cells of lymphocyte origin in the lesions. However, infection of lymphocytes in the peripheral blood has been previously observed (Chapter 5).

Comparing the distribution of the mild lesions to the advanced lesions, in this study, it appears that the spleen is one of the first organs colonized by neoplastic histiocytic cells. Early lesions in the spleen, in some birds, were relatively subtle and could easily be mistaken for an inflammatory (histiocytic) splenitis. The esophagus, crop, esophageal/proventricular junction, and lamina propria of various portions of the intestine appear to be common locations for neoplastic cell colonization as the disease progressed. Interpretation of the mild neoplastic changes in the esophagus was complicated by concurrent esophagitis believed to be associated with the *Capillaria* sp. Infection found even in controls not infected with REV. Massive spleen and liver involvement developed at the terminal stages following infection. This distribution of spleen and liver lesions is consistent with that reported in several other species (Dren, 1988b; Grimes and Purchase, 1973; McDougall, 1978; Witter, Smith, and Crittenden, 1981; Witter, Sharma, and Fadly, 1986), although the consistent combination of splenic, liver, and digestive lesions is somewhat unique.

Two experimentally infected birds developed papillomatous lesions of the proventriculus. This is an unusual lesion and its relationship to the REV infection remains unclear, but could be a consequence of immunosuppression. Mucosal papillomas of the digestive tract of parrots are believed to be caused by the Psittacid herpesvirus (Styles, 2004). These tissues were not tested for herpesviruses, but if this

type of lesion is seen again in birds with REV, further investigation into its etiology may be warranted.

In the present study, REV infection at three different doses resulted in 100% infection among all groups that received the virus. A minimum infectious dose of $TCID_{50}$ 200 was shown to be capable of causing infection as well as generating the disease. This is the lowest dose of REV reported to cause infection in any avian species to date.

CHAPTER VII

DEVELOPMENT OF A DNA VACCINE

INTRODUCTION

The Attwater's prairie chicken (*Tympanachus cupido attwateri*), a subspecies of *Tympanachus cupido*, has faced several obstacles in its survival as a species. The loss of habitat and over hunting have led to a decimation in the population resulting in its endangered status. Currently, Attwater's prairie chickens are housed in captive populations at several facilities within Texas, in hopes of restoring their numbers and maintaining the subspecies. Repopulation of the species has been complicated by the prevalence of REV infection in these birds, first reported by Drew et al. (1998) at Texas A&M University. Subsequent infections have been detected at several breeding facilities in Texas.

Reticuloendotheliosis virus infection in the Attwater's prairie chicken results in a neoplastic disease commonly seen in various other avian species (Witter, 2003). Although not considered a problem of concern within the poultry industry (Witter, 2003), mortality rates observed in prairie chicken populations have been notably high, in some instances up to 100% (personal communication). The current survival of these birds in breeding facilities relies on minimizing the spread of infection within a population and ultimately developing an effective strategy of control. Vaccines provide

a suitable method of prevention of infection as well as a therapeutic option for reduction of viral loads in infected birds.

The nature of retroviruses to incorporate their genomes into host chromosomes precludes the use of live, attenuated vaccines. A live vaccine would likely persist as a provirus with an opportunity to mutate to a more pathogenic strain. Alternatively, subunit protein vaccines are safe but are not effective at inducing lasting cellular immunity. However, gene vectored vaccines such as DNA expression plasmids are ideal candidates because they are the safest vaccines that also induce lasting cellular immunity to infectious diseases (Donnelly, 1997). DNA vaccines have been effective in providing complete or partial protection from several avian viral infections including avian influenza (Fynan, 1993; Kodihalli, 1997), Newcastle disease virus (Sakaguchi, 1996), infectious bursal disease virus (Chang, 2001; Fodor, 1999), and infectious bronchitis virus (Seo, 1997).

In this chapter, the efficacy of a DNA eukaryotic expression vector containing sequences of REV gag and env genes was investigated for its ability to decrease viral loads to undetectable levels in REV infected Attwater's prairie chickens. Whereas protection against infection is critical in eliminating the virus in captive populations of Attwater's, experimental vaccination and infection of endangered birds is not an option. Alternatively, reduction in viral loads of naturally infected birds by a therapeutic vaccine would aid in minimizing spread of virus within a population. Additionally, identification of an effective therapeutic vaccine would be a step in the direction of testing a prophylactic vaccine in the future.

MATERIALS AND METHODS

Birds

A group of 19 Attwater's prairie chickens at Fossil Rim Wildlife Center (FRWC) testing positive for REV infection by nested PCR analysis were housed outside in a pen of approximately 300 m^2. The pen was surrounded on all sides and top by wire mesh.

Vaccine construction

Three PCR amplification products within the gag gene and one PCR amplification product within the env gene were made to construct the expression plasmids. All three amplification products within the gag were of varying lengths extending to the stop codon. The env amplification product encompassed 1,330 base pairs starting 150 base pairs down stream of the env start codon. Primers and amplification products are further described in Table 9.

Two µl of genomic DNA template from REV infected chicken embryo fibroblast cells were added to a 50 µl reaction containing 36.75 µl water, 5 µl 10X Mg free reaction buffer (Promega, Madison, WI), 3 µl 10 mM $MgCl_2$, 1 µl 10 mM dNTP mix, 1 µl (10 pmol) of forward primer, 1 µl (10 pmol) reverse primer and 0.25 µl Taq polymerase (Promega, Madison, WI). Samples were incubated at 95º C for 5 minutes,

Table 9. Primers used for PCR amplification of inserts used in each clone.

Clone	Fwd Primer	Rev Primer	Size (bps)
12-4	REVF 1292	SQREVgag1	1194
15-2	REVF 1574	SQREVgag1	912
18-2	REVF 1853	SQREVgag1	633
4-3	REVF 6157	REVR7487	1331

then cycled at 95º C for 30 seconds, 55º C for 30 seconds and 72º C for 2 minutes for 40 cycles. Samples were incubated at 72º C for 7 minutes, before storing at 4º C. PCR products were purified with a GenElute PCR clean-up kit (Sigma, St. Louis, MO).

Amplification products were cloned in frame into the pcDNA 3.1/V5-His TOPO TA Expression vector (Invitrogen, Carlsbad, CA) according to manufacturer's protocol and plated on LB plates with 50 μg/ml ampicillin. Plates were incubated overnight at 37° C and colonies were pulled the following day and inoculated into 3 ml LB broth with 50 μg/ml ampicillin and shaken overnight at 37° C. Plasmid was purified from each culture using the GenElute Plasmid DNA Miniprep Kit (Sigma, St. Louis, MO) and positive clones were identified by PCR amplification as described above. Clones were sequenced to verify that the amplification products were in frame and no stop codons were present. Positive clones described in Table 1 were designated as 4-3 (*env*), 12-4 (*gag*), 15-2 (*gag*) and 18-2 (*gag*).

Transfection and western blot

The DNA-based expression constructs (2 μg) were transfected into 293T cells in a 6-well plate with PolyFect transfection reagent (QIAGEN, Valencia, CA). Two days after transfection, the supernatant was collected and the cells were lysed with lysing buffer (50 mM Tris-HCl, 1% Triton X-100, 0.15% sodium dodecyl sulfate, 150 mM NaCl, and 20 mM EDTA). Both supernatants and cell lysates were subjected to sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and transferred onto a

nitrocellulose membrane (0.45 um pore size, Bio-Rad, CA). After blocking the membrane with 0.5% casein in TBS (10 mM Tris-HCl, pH 7.5, 150 mM NaCl) for 1 hour at room temperature (RT), the membranes were washed three times with TBS-T (TBS containing 0.1% Tween 20), reacted to rabbit anti-REV-gag polyclonal serum for 1 hour at RT, and then incubated with goat anti-rabbit IRDye800 (Rockland Immunochemicals, PA) for 1 hour at RT. The blots were washed with TBS and the protein bands were detected using an Odyssey Infrared Imaging System (LI-COR, Lincoln, NE).

Collection of blood

Two milliliters (ml) of blood were collected via jugular venipuncture at each testing period. Blood was diluted 1:1 with Alsever's solution and under-laid with 2 ml of Histopaque (Sigma, St. Louis, MO). Cells were spun at 400 x gravity for 30 minutes. Peripheral blood mononuclear cells (PBMC) were collected from the suspended layer and washed 2 times in 10 ml PBS with 0.02% BSA and 0.02% sodium azide (PBA).

PCR diagnosis of REV infection

Genomic DNA was extracted from PBMC from 0.5 ml of blood using the GenElute Mammalian Genomic DNA Miniprep kit (Sigma, St. Louis, MO) and stored at -20 °C. Two ml of genomic DNA template were added to a 50 microliter (µl) reaction

containing 36.75 µl water, 5 µl 10X Mg free reaction buffer (Promega, Madison, WI), 3 µl 10 mM $MgCl_2$, 1 µl 10 mM dNTP mix, 1 µl (10 pmol) of forward primer, 1 µl (10 pmol) reverse primer and 0.25 µl Taq polymerase (Promega, Madison, WI). Samples where held at 95º C for 5 minutes, then cycled at 95º C for 30 seconds, 55º C for 30 seconds and 72º C for 2 minutes for 40 cycles. Samples were then held at 72º C for 7 minutes before storing at 4º C. Primers chosen using OLIGO 4.0 software used for first step amplification PCR were REVR3559 (TGCCACCCGAGACTTACTCA) and REVR5306 (CCCAGCCCGAGAATGTGTCTAC) within the polymerase gene.

Following first step amplification PCR, positive samples yielded a 1,747 base pair (bp) product. Two µl of the product from the reaction following the first step amplification were added to a second 50 µl reaction (prepared just as the first and run under the same cycle conditions). Primers used for the second step amplification of the nested PCR reaction were REVF3559 and REVR4138 (CTGCCCGAAGGTAAGTTTAGAG). Following the second step amplification, positive samples yielded a 869 bp product. Positive controls of genomic DNA extracted from REV infected DF-1 cells, as well as negative controls containing no DNA, were included in all reaction sets.

Five µl of PCR products following both first and second step amplifications were electrophoresed on 1% agarose gels in tris-acetate buffer (TAE; 0.4 M tris-acetate, 0.001 M EDTA) with 0.5 µg/µl ethidium bromide. Bands resulting in either first or second step amplification reactions indicated samples positive for REV.

Vaccination timeline

Birds were designated into 3 treatment groups. All DNA was diluted in sterile PBS. One day prior to the first inoculation, all birds received 200 µl 0.25% bupivacaine in each side of the breast to aid in stimulation of an immune response. All birds received 2 inoculations intramuscularly (im) of 200 µl each in the breast muscle at the site of bupivacaine injection. Group 1 consisted of 6 birds receiving sham inoculations of sterile PBS on both sides of the breast. Group 2 received inoculations of 20 µg empty plasmid vector pcDNA 3.1 containing no viral genes in each side of the breast. Group 3 received pcDNA3.1 with the gag insert in the right side of the breast and pcDNA3.1 with the env insert in the left side of the breast. Birds were vaccinated 3 times with one week intervals between inoculations. Birds in groups 2 and 3 received 20 µg DNA on each side of the breast in each of the first 2 weeks and 40 µg DNA on each side of the breast in the third inoculation. Blood was drawn on week 0 (first inoculation, week 2 (third inoculation) and week 4 for analysis of REV infection by PCR.

RESULTS

Confirmation of protein expression

All 3 plasmids expressing varying sizes of the gag polypeptide were transfected into cells and screened for expression with polyclonal rabbit anti-gag antibody by western blot (Figure 26). Bands represent the different gag constructs of 1,194 base pairs (clone

12-4), 912 base pairs (clone 15-2) and 633 base pairs (clone 18-2). Expression of clones 12-4 and 15-2 was unclear as faint bands of the appropriate size appear to be present. However, a band of about 25 kilodaltons representing clone 18-2 was clearly present confirming expression of the polypeptide in this construct. Confirmation of expression by the env construct was not obtained as available antibodies to the REV envelope were not effective in recognizing the expressed envelope protein.

Vaccination of infected birds resulted in no decrease of viral load

Prior to vaccination, all birds were screened for REV by both single-step and nested PCR. Infected birds were assigned to three different treatment groups receiving PBS only (group 1), pcDNA 3.1 expression plasmid without an insert (group 2) and pcDNA 3.1 expression plasmid containing both *gag* and *env* genes (group 3). 5 of 6 birds in each of the three groups were positive by single-step PCR amplification. All birds in groups 1 and 3 and 5 of 6 birds in group 2 were positive by nested PCR prior to vaccination. Based on expression of the gag polypeptide (Fig. 26), clone 18-2 was chosen for inoculation in the vaccine study. Despite confirmed expression of the env protein, clone 4-3 was used for inoculation of an envelope expression vector.

Following inoculation with the vaccine at 2 and 4 weeks post inoculation (pi), percentages of birds positive for REV in the vaccine treated group (group 3) by single-step amplification increased. Nested PCR amplification revealed no change in positive birds following inoculation with the gag and env expression constructs (Table 10).

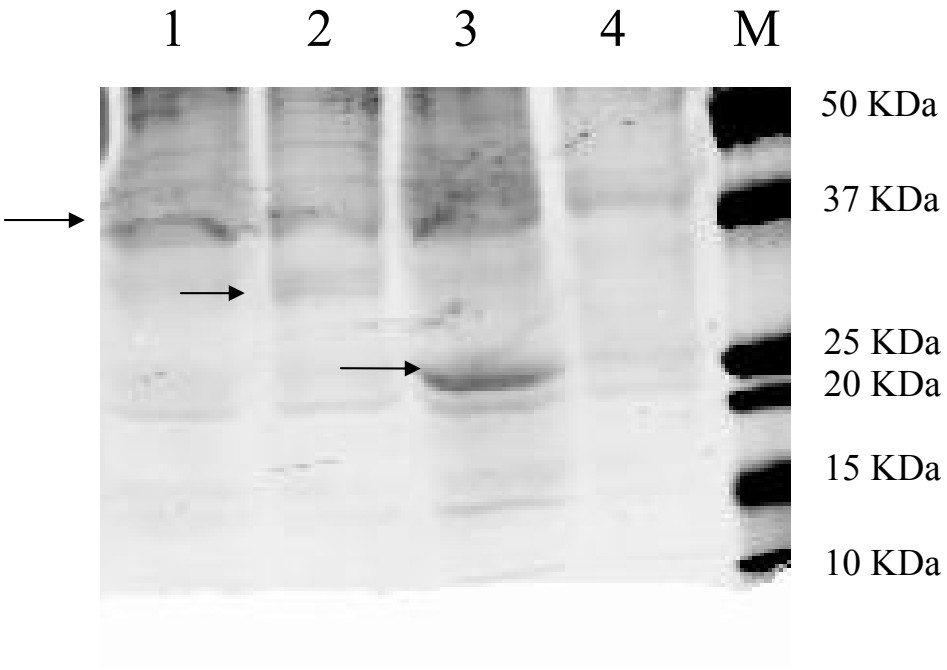


Figure 26. Western blot of gag polypeptides expressed in eukaryotic cells following transfection. Lane 1 represents clone 12-4, lane 2 represents clone 15-2 and lane 3 represents clone 18-2.

Table 10. Percentage positive birds by single-step and nested PCR at each week.

	Week 0		Week 2 pi[a]		Week 4 pi[a]	
	Single-step	Nested	Single-step	Nested	Single - step	Nested
Group 1 (PBS) n=6	83.3	100	100	100	100	100
Group 2 (DNA control) n=6	83.3	83.3	80	100	83.3	100
Group 3 (REV gag and env) n=6	83.3	100	100	100	100	100

[a]post infection

No discernable changes in REV positive birds were seen in control groups 1 or 2 receiving PBS and empty plasmid DNA.

DISCUSSION

Therapeutic immunization against REV in naturally infected Attwater's prairie chickens was not achieved using eukaryotic expression vectors containing inserts of REV gag and env genes. Despite the absence of a decrease in detectable viral levels exhibited by consistently positive single-step PCR amplification products of proviral DNA in all groups, further modifications may reveal a suitable vaccine strategy.

A therapeutic vaccine would be useful if its dose resulted in a decrease of viral loads within infected birds, thus minimizing potential spread of the virus within a captive flock due to shedding. Additionally, a reduction in viral shedding is associated with decreased vertical transmission rates (McDougall, 1980; Motha, 1984; Witter, 2003; Witter, Smith, and Crittenden, 1981), which could allow infected dams to produce uninfected offspring capable of remaining in the general population. The problem with a therapeutic vaccine is that it targets cells of the immune system to provide protection against the virus. However, these cells are permissive to infection by REV, and may well be infected by the virus in infected birds, thus minimizing the efficaciousness of this vaccine strategy.

The ultimate goal of our REV research in the Attwater's prairie chicken is to develop a vaccine capable of providing prophylactic protection. Although the present

study did not result in therapeutic protection, prophylactic protection in uninfected birds using this strategy has not been determined. One potential strategy that could circumvent the issue of infecting endangered birds with REV is to inoculate a large number of REV negative birds in captive populations using the same group designations described in this study. A survey over time of birds becoming infected with REV in each group may reveal the potential efficacy of this vaccine. A more direct approach would rely on breeding and using Attwater's/greater hybrids in a vaccine study, in which birds would be directly infected with REV following vaccination.

Incorporation of immunostimulatory molecules and fusion proteins that promote appropriate processing may be added to the plasmid expression vectors that could enhance immunity (Donnelly, 1997; Sykes, 2002; Xiang, 1995). IFN-γ and IL-12 have been implicated in immune enhancement in mammals (Krieg, 2001) and have been cloned and sequenced for chickens (Degen WG, 2004; Digby, 1995). IL-15 may also be a worthwhile consideration in booster inoculations because it is known, in mammals, to enhance the memory immune response required for challenge protection (Zhang, 1998).

CHAPTER VIII

SUMMARY AND CONCLUSIONS

The focus of this study was to develop a better understanding of REV infection in the endangered Attwater's prairie chicken in the ultimate pursuit for a method of protection. Several aspects of this virus were investigated including its origin and strain classification, target cell of infectivity and pathogenesis in prairie chickens. Finally, the efficacy of a therapeutic vaccine was tested for its ability to reduce detectable virus levels in infected birds.

In order to investigate the origin of the REV strain infecting the prairie chickens, two Texas prairie chicken isolates were obtained, along with prototype viruses REV-A, SNV and CSV, and two REV isolates collected from poxvirus lesions on Texas domestic chickens. The prairie chicken isolates were isolated in 1994 and 2003 and both domestic chicken isolates were isolated in 2003. A region spanning the entire polymerase through most of the envelope were sequenced and compared in one of the prairie chicken isolates (PC-R92) and REV-A showing 98% identity. Subsequent sequencing of the polymerase and envelope of the remaining strains showed that nucleotide and amino acid identities among all viruses ranged from 90-99%. SNV, a duck virus, proved to have the least sequence homology with all the other viruses and clustered within its own separate group. The CSV, Texas domestic chicken and prairie chicken isolates had the greatest homology with each other among all of the viruses with the REV-A proving to be slightly less homologous. Interestingly, no greater homology was seen between the two

prairie chicken isolates and the other isolates. The prairie chicken isolate from 1994 was no more different from the Texas chicken isolates than the prairie chicken isolate from 2003. All of these isolates were equally as homologous to the CSV prototype virus isolated in 1969. The high degree of homology among all of these viruses indicates that the virus currently infecting the Texas domestic chickens and prairie chicken flocks originated from the CSV strain. Additionally, it appears that little change has occurred in this virus over decades, which is typically uncommon in retroviruses.

Several possibilities exist to explain this low level of variation. Selection pressures on the infectious virus may be such as to not tolerate minor mutations in the viral proteins. Alternatively, the fidelity of the reverse transcriptase may be much higher than that of other retroviruses, resulting in greater preservation of the genome sequence. However, the association of REV with poxvirus could also present a plausible scenario. As the REV genome has been shown to be incorporated into the genome of the poxvirus, these natural poxviruses vectors may be responsible for transmission of REV from flock to flock allowing the incorporated REV to remain unchanged.

The association of REV with the pox virus has been well documented (Hertig et al., 1997; Singh, Schnitzlein, and Tripathy, 2003). Outbreaks of REV within captive prairie chicken flocks have often coincided with outbreaks of pox lesions within the flock (personal observation). The reported integration of the chicken REV genome into the poxvirus genome may explain transmission of the virus among flocks. Sequencing data in this study support such a mechanism as the Texas domestic chicken REV isolates

were collected from birds infected with poxvirus and were highly identical to the prairie chicken.

The potential mechanism of REV transmission through a poxvirus vector could be further supported by the higher incidence of REV infection occurring in summer months. Mosquitoes have been indicated as a possible means of mechanical transmission of REV although it has not been conclusively shown. However, poxviruses are commonly transmitted by mosquitoes, which may explain this correlation making this mechanism a plausible scenario to explain the current transmission of REV among flocks. Further characterization of the poxvirus infecting prairie chicken flocks and understanding its involvement in REV transmission are necessary, but may provide a target for a potential vaccine.

Reagent development was a critical first step in undertaking research focusing on REV infection of the Attwater's prairie chicken. Reagents recognizing prairie chicken antigens were not commercially available and antibodies recognizing REV antigens were not easily obtainable. Specifically, rabbit polyclonal antibodies recognizing both purified REV gag polypeptide and prairie chicken IgY were developed and purified for use in antigen and antibody ELISAs. The polyclonal rabbit anti-prairie chicken IgY was used for development of an antibody capture ELISA and was successful in identifying the immunoglobulin. Interestingly, although antibodies recognizing chicken IgY were unable to cross react with the prairie chicken IgY, the polyclonal rabbit anti prairie chicken IgY did recognize chicken immunoglobulin, thus showing some antigenic similarities between the two species. The antibody capture ELISA will prove useful in

investigating antibody responses of prairie chickens to not only REV infection, but other diseases as well, in order to help determine their immune response to infection.

An antigen ELISA using the polyclonal rabbit anti-REV gag was developed for detection of virus and was successfully used for quantitation of viral titers in combination with a $TCID_{50}$ assay using chicken embryo fibroblast cell culture. Although the antibody recognized antigens from prairie chicken REV isolates, its ability to recognize isolates of other REV strains or isolates from other avian species has not been investigated.

The low level of mutation of viable virus may have implications on the cross reactivity of polyclonal antibodies developed against the REV gag polypeptide. Although these antibodies have not been tested for their ability to cross react with other isolates or the REV-A, CSV, and SNV prototypes, the high level of conservation of the genome within the pol and env genes suggests that cross reactivity is quite possible. Additionally, this data may aid in vaccination strategies as a single vaccine to protect against the various strains as the relationship among the viruses is so close.

A strategy for analyses of lymphocytes in avian species was developed as a part of this study to further investigate the implications of REV infection in the prairie chickens. The monoclonal antibody, K55, developed as a pan leukocyte marker in chickens (Chung, Lillehoj, and Jenkins, 1991) was found to cross react with prairie chicken cells. This antibody was used to segregate leukocyte populations from contaminating thrombocytes for flow cytometric analyses. An exclusively lymphocyte population was identified in PBMC labeled with K55, which allowed for further gating strategies with

various markers, including CD4 and CD8. The implications of this gating strategy allow for investigations into the fluctuation of these lymphocyte subsets with greater accuracy, as the high level of thrombocyte populations are excluded from the data set. This strategy would prove useful in evaluating lymphocyte subset fluctuations in not only prairie chickens, but chickens as well as other avian species. A third population of cells, presumed to be monocytes, were also labeled with K55 at a higher intensity than the lymphocyte population. However, the K1 monoclonal antibody described as specific for a common thrombocyte and monocyte marker (Kaspers, Lillehoj, and Lillehoj, 1993), did not label cells within this population. Although further investigation into the identity of these cells is required, evaluation of monocyte fluctuations using this strategy is also possible.

The K55 gating strategy was used in combination with intracellular labeling using the polyclonal rabbit anti REV gag antibody, which was generated with purified recombinant polypeptide, in order to identify lymphocyte subsets that support REV replication. Cells were labeled with K55 conjugated to biotin, and either CD4 or CD8 monoclonal antibodies conjugated to PE. Subsequently, cells were permeablized and labeled with the rabbit anti REV gag conjugated to FITC and analyzed by flow cytometry. High percentages, that is greater than 75%, of either CD4 and CD8 lymphocytes from the infected birds in this study supported REV replication. In contrast, negligible staining was seen in lymphocyte subsets from uninfected birds. The majority of K55 – high population, presumed to be monocytes, and the thrombocyte population were also found to express the REV gag antigen. No population labeled with

the K55 was identified that did not express the antigen. Because viral antigen was not detected in control leukocytes prepared from uninfected birds, the antibody specifically identified infected cells. Therefore, these studies indicated that a majority of leukocytes in chronically infected birds could be productively infected with REV. This high rate of infection indicated that leukocytes, in general, are permissive to infection by REV. Additionally, the large percentages of cells infected may be the result of neoplastic proliferation induced by REV infection.

The lack of a B cell marker capable of cross-reacting with prairie chicken B cells, prevented investigation of this population. However, the presence of T cell, as well as B cell, markers in tumors induced by REV infection (Cooper, 1991; Nazerian et al., 1982) in domestic chickens implies the strong possibility that B cells may also be infected by REV in the prairie chicken. Future investigation should further characterize the nature of the target cells of REV infection are required.

The extent of infection in the captive flocks provided a continuous source of infected birds. However, studies following experimental infection allow for controlled conditions necessary to determine the pathogenesis of REV in the prairie chicken. A dose response infectivity study was conducted on hybrid greater/Attwater's prairie chickens using three different doses of the prairie chicken isolate PC-R92: $TCID_{50}$ 200, $TCID_{50}$ 1000, and $TCID_{50}$ 5000. The nested PCR diagnostic test was developed because the pathogenesis study indicated that the single step PCR was not sensitive enough to identify early infection. The more sensitive assay was capable of identifying infection in all birds given the virus as early as 2 weeks p.i. In comparison with the

single round amplification PCR test, 100% of the birds tested positive by the nested PCR at all time points whereas the single round amplification resulted in only 62% positive results over the course of the study, thus showing the greater effectiveness of the nested PCR in identifying infected birds. Genomic DNA from PBMC was used as a template in all birds tested. A standardization assay utilizing a bacterial recombinant plasmid encoding a segment of the pol gene as template, determined the sensitivity of the nested PCR assay was at least 10^2 copies of DNA per reaction.

The dose response pathogenesis study was conducted over a 37 week period, however, birds in the lowest dosage group were euthanized at 20 weeks p.i. Neoplasias appeared to develop around 20 weeks p.i., as all birds in the low dosage group were affected, except one which died at 8 weeks p.i. from a *Pseudomonas* infection of the lung. Lesions in these birds were commonly found in the spleen, but were absent in the liver suggesting that the spleen is one of the first organs colonized by neoplastic cells. Neoplastic lesions were present in all birds in both of the higher dosage groups, and were most commonly found in the liver and spleen, however, lesions at the esophageal/proventricular junction were also common. The terminal stages of the disease were characterized by massive involvement of the spleen and liver. The development of neoplasia in the lowest dosage group of birds receiving $TCID_{50}$ 200 of virus is the lowest dose of REV reported to cause infection in any avian species.

The low level of mutation attributed to this virus based on sequencing data indicates that a single vaccine may prove useful for not only protecting against the prairie chicken isolate, but potentially many or all REV strains. For safety reasons, a

cDNA vaccine was developed incorporating *gag* and *env* genes cloned into a eukaryotic expression vector. These recombinant plasmids were administered i.m. in the breast of naturally infected Attwater's prairie chickens for 3 consecutive weeks to test as a therapeutic vaccine. In the absence of experimental birds, the goal of the study was to determine the effectiveness of the vaccine as a therapeutic method of reducing viral load. One day prior to the first inoculation, birds were given an inoculation of the anesthetic bupivacaine in the breast muscle to aid in the stimulation of an immune response to the targeted area of vaccination. Vaccinated birds were bled at two week intervals and tested for REV by both single step amplification and nested PCR of the genomic DNA from PBMC. Although the PCR assays are only crudely quantitative, a decrease in detectable virus levels was not observed and all birds testing positive following a single step PCR amplification remained positive with this test for the duration of the study.

Traditional challenge vaccine trials using uninfected birds are necessary to determine the potential efficaciousness of these recombinant plasmids in preventing infection with REV. As a low dose of infection has been determined based on our dose response infectivity study, a similar study should be done in determining the prophylactic effect of this vaccine. Additional modifications to the plasmid may also aid in its efficacy. Sequences of chicken immune enhancers, such as IL-2 and IL-15, are now known and could be cloned into the vector, thus aiding in stimulating the immune response.

The effect of REV infection on prairie chickens is clearly serious, as the duration of survivors among infected birds is typically short. This severity of infection effect is

further indicated by the high percentage of lymphocytes that can be infected by REV as seen by intracellular labeling of gag proteins with flow cytometry. Although this study only examined T lymphocyte infection, the B lymphocytes are very likely also targets of REV infection in the prairie chicken, since the percentage of infected lymphocytes exceed what are accountable by T cells alone. Additionally, the involvement of B cell tumors have been described in REV infected poultry. Flow cytometry studies also indicated that a majority of monocytes are infected. Neoplastic cells from lesions resulting from REV infection in our pathogenesis study most closely resembled histiocytic cells, which are derived from monocytes, further implicating this population as a potential target of infection. Future investigation of other cell types involved in the immune system as potential targets of REV is necessary to understand the full range of cells infected by REV and thereby understand the impact this virus has on the prairie chicken immune system. This study has shown that REV infection results in a severe disease in the Attwater's prairie chicken. The implications of this disease and its effect on these birds cannot be ignored if repopulation and maintenance of this species is to be achieved in captive populations.

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PUBLICATIONS

1. Bohls, R. L., Smith, R., Ferro, P. J., Silvy, N. J., Li, Z., and Collisson, E. W. 2006. The use of flow cytometry to discriminate avian lymphocytes from contaminating thrombocytes. Developmental and Comparative Immunology. (in press).

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